

# **Regulation of human fibroblast insulin-like growth factor (IGF)-binding proteins by IGF-1 and cytokines, mechanisms of action and effects upon IGF bioactivity**

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**REGULATION OF HUMAN FIBROBLAST INSULIN-LIKE  
GROWTH FACTOR (IGF)-BINDING PROTEINS BY  
IGF-I AND CYTOKINES, MECHANISMS OF ACTION  
AND EFFECTS UPON IGF BIOACTIVITY.**

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**A thesis submitted for the degree of  
Doctor of Philosophy in the Faculty of Medicine  
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## ABSTRACT

The insulin-like growth factors, IGF-I and IGF-II, are ubiquitous polypeptide molecules that have mitogenic and metabolic actions in a wide variety of cell types, and consequently play a major role in mammalian growth and development. Unlike many other peptide hormones, IGF levels and their bioactivity are highly dependent upon the secretion of a family of six specific binding proteins, named IGFBPs. In this thesis, we have developed a normal human fibroblast *in vitro* cell culture model to investigate both factors that affect IGFBP secretion, the mechanisms behind such regulation, and also the effect of IGFBP modulation upon subsequent IGF-I mitogenic activity.

Firstly, we have examined the possible role that the recently discovered IGFBP proteases may have in determining the effect of IGF-I on IGFBP-3 abundance in fibroblast conditioned media. We show that IGF-I increased IGFBP-3 when assessed by ligand blotting, but did not increase immunoreactive IGFBP-3, a discrepancy that could be explained by further data showing an inhibitory effect of IGF-I on the activity of the fibroblast IGFBP-3 protease. Thus, IGF-I protection of IGFBP-3 from enzymatic degradation may help explain the post-transcriptional, non-receptor mediated 'stimulation' of IGFBP-3 by IGF-I in these cells.

We also show for the first time the ability of a number of cytokines to regulate IGFBP secretion, perhaps indicating a novel pathway of communication between these immune cell molecules and the IGFs. The inflammatory cytokine interleukin 1 $\beta$  (IL-1 $\beta$ ) inhibited Hs68 fibroblast IGFBP-3 secretion by down-regulating its gene expression, whilst tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) had a similar inhibitory effect on IGFBP-3 and IGFBP-4 but acted via a post-transcriptional mechanism. The exact nature of the TNF $\alpha$  effect remains to be determined as no evidence was found to suggest TNF $\alpha$  increased IGFBP protease activity, or that TNF $\alpha$  removed IGFBPs from the conditioned media by increasing the proportion immobilised on the cell surface. The inhibition of IGFBP secretion by TNF $\alpha$  was observed to have marked effects upon the mitogenic activity of IGF-I in these cells, with a five-fold increase in sensitivity to the growth factor seen in a novel cytochemical bioassay.



Inhibition of fibroblast IGFBP secretion appeared to be restricted to certain cytokines as IL-6 had no effect, whilst high doses of interferon gamma abolished the TNF $\alpha$  effect. Conversely, transforming growth factor  $\beta$  (TGF $\beta$ ) directly stimulated fibroblast IGFBP-3 secretion, via an increase in gene expression, and subsequently resulted in the reduction in the mitogenic activity of IGF-I.

These data indicate that a variety of mechanisms can be employed by a number of factors to elicit changes in fibroblast IGFBP secretion, and that these changes may have direct consequences in determining IGF-I bioactivity. Such is the importance given to the IGFs in maintaining normal somatic growth, changes in IGFBP secretion may contribute to the altered cellular growth and metabolism seen associated with cytokines in conditions as diverse as chronic infection, rheumatoid arthritis, cancer and the wound healing process.



TABLE OF CONTENTS

Abstract ..... 1

Table of Contents ..... 3

List of Figures ..... 6

List of Tables ..... 9

Attribution ..... 10

Acknowledgements ..... 11

Dedication ..... 12

Abbreviations ..... 13

CHAPTER 1 : INTRODUCTION ..... 15

Section A. IGF Characterisation ..... 16

    1.1. Historical Perspectives ..... 16

    1.2. IGF Peptide Structure ..... 17

    1.3. IGF Gene Structure ..... 18

    1.4. IGF Function ..... 21

    1.5. Control of IGF secretion ..... 24

    1.6. Cell Membrane IGF Receptors ..... 25

Section B. The Insulin-like Growth Factor Binding Proteins	30
1.7. Insulin-like Growth Factor Binding Protein-3	30
1.8. Insulin-like Growth Factor Binding Protein-1	38
1.9. Insulin-like Growth Factor Binding Protein-2	41
1.10. Insulin-like Growth Factor Binding Protein-4	43
1.11. Insulin-like Growth Factor Binding Protein-5	45
1.12. Insulin-like Growth Factor Binding Protein-6	46
Section C. The Cytokine Family	49
1.13. Transforming Growth Factor $\beta$	49
1.14. Tumour Necrosis Factor $\alpha$	51
1.15. Interleukin 1 and 6	54
Section D. Aims of Thesis	57
<b>CHAPTER 2 : MATERIALS and EQUIPMENT</b>	59
<b>CHAPTER 3 : METHODS</b>	69
3.1. Cell Culture	70
3.2. Western Ligand Blotting (WLB)	72
3.3. IGFBP-3 Radioimmunoassay (RIA)	79
3.4. Molecular Biology	84
3.5. IGFBP-3 Protease Analysis	101
3.6. Fast Phase Liquid Chromatography	104
3.7. IGF-I Cell Binding Assay	105
3.8. IGF Bioassay	107
3.9. Statistical Analysis	113

<b>CHAPTER 4 : MECHANISMS OF IGF REGULATION OF IGFBP-3 IN VITRO.</b>	118
<b>CHAPTER 5 : REGULATION OF FIBROBLAST IGFBPs BY TUMOUR NECROSIS FACTOR <math>\alpha</math>.</b>	137
<b>CHAPTER 6 : REGULATION OF FIBROBLAST IGFBPs BY INTERLEUKIN 1 AND 6.</b>	156
<b>CHAPTER 7 : REGULATION OF FIBROBLAST IGFBPs BY TRANSFORMING GROWTH FACTOR <math>\beta_1</math>.</b>	169
<b>CHAPTER 8 : THE EFFECT OF ENDOGENOUS IGFBP REGULATION UPON IGF-I BIOACTIVITY.</b>	183
<b>CHAPTER 9 : DISCUSSION</b>	201
Section A: The effect of IGF-I upon IGFBP-3.	
9.1. A role for protease modulation ?	202
Section B: Cytokine regulation of IGFBPs.	
9.2. The relationship of $TNF\alpha$ and IL-1 to the IGFs.	206
9.3. The relationship of $TGF\beta$ to the IGFs.	211
Section C: Future objectives.	214
<b>CHAPTER 10 : REFERENCES</b>	215



## LIST OF FIGURES

<b>Figure 1.i.</b> Schematic representation of IGF-I DNA, mRNA and precursor proteins.....	19
<b>Figure 1.ii.</b> Schematic representation of IGF-II DNA, mRNA and precursor proteins.....	20
<b>Figure 1.iii.</b> Amino acid sequences of human IGFBP-1 to IGFBP-6.....	31
<b>Figure 3.i.</b> Size exclusion chromatographic profile of <sup>125</sup> I radiolabelled IGF-I.....	78
<b>Figure 3.ii.</b> Serial dilution of Hs68 fibroblast conditioned medium in the IGFBP-3 radioimmunoassay.....	82
<b>Figure 3.iii.</b> The effect of Hs68 fibroblast protease on the IGFBP-3 radioimmunoassay.....	83
<b>Figure 3.iv.</b> Schematic representation of the human IGFBP-3 cDNA probe construction.....	85
<b>Figure 3.v.</b> Size assessment of the IGFBP-3 cDNA clone following excision from plasmid.....	91
<b>Figure 3.vi.</b> Analysis of IGFBP-3 cDNA following purification from agarose gel and plasmid DNA.....	93
<b>Figure 3.vii.</b> Size exclusion chromatographic profile of radiolabelled IGFBP-3 cDNA.....	95
<b>Figure 3.viii.</b> Chemical structure of MTT and its reduction to a coloured formazan.....	109
<b>Figure 3.ix.</b> Time-course of MTT formazan production by Hs68 fibroblasts.....	111
<b>Figure 3.x.</b> The positive correlation between MTT formazan production and Hs68 fibroblast cell density.....	112
<b>Figure 4.i.</b> Western ligand blot of Hs68 cell IGFBPs in response to IGF-I and LongR3-IGF-I.....	123

<b>Figure 4.ii.</b> The effect of IGF-I and LongR3-IGF-I on IGFBP-3 immunoreactivity in Hs68 cell conditioned media.....	125
<b>Figure 4.iii.</b> Northern blot of Hs68 cell IGFBP-3 mRNA in response to IGF and related peptides.....	126
<b>Figure 4.iv.</b> The effect of IGF-I on Hs68 cell IGFBP-3 protease activity during 3 hour incubations.....	127
<b>Figure 4.v.</b> The effect of IGF-I on Hs68 cell IGFBP-3 protease activity during 6 hour incubations.....	129
<b>Figure 4.vi.</b> The effect of IGF-I on Hs68 cell IGFBP-3 protease activity during 24 hour incubations.....	130
<b>Figure 4.vii.</b> Size exclusion chromatographic profile of <sup>125</sup> I-IGFBP-3.....	131
<b>Figure 4.viii.</b> Size exclusion chromatographic profile of <sup>125</sup> I-IGFBP-3 following treatment with Hs68 protease.....	132
<b>Figure 4.ix.</b> Size exclusion chromatographic profile of <sup>125</sup> I-IGFBP-3 following treatment with Hs68 protease in the presence of EDTA.....	134
<b>Figure 5.i.</b> Western ligand blot of Hs68 cell IGFBPs following treatment with TNF $\alpha$ .....	141
<b>Figure 5.ii.</b> Inhibition of Hs68 cell immunoreactive IGFBP-3 by TNF $\alpha$ .....	142
<b>Figure 5.iii.</b> The effect of PGE <sub>2</sub> and indomethacin on Hs68 cell IGFBP-3.....	144
<b>Figure 5.iv.</b> The effect of interferon gamma on Hs68 cell IGFBP-3.....	145
<b>Figure 5.v.</b> Northern blot of Hs68 cell IGFBP-3 and -4 mRNA following treatment with TNF $\alpha$ 24 hours.....	147
<b>Figure 5.vi.</b> Northern blot of Hs68 cell IGFBP-3 and $\beta$ -actin mRNA following treatment with TNF $\alpha$ for 2,4,6,8,12 hours.....	148
<b>Figure 5.vii.</b> The effect of 24 hour TNF $\alpha$ treatment upon Hs68	



cell IGFBP-3 protease activity.....	149
<b>Figure 5.viii.</b> Alteration of Hs68 cell <sup>125</sup> I-IGF-I binding sites by TNF $\alpha$ treatment.....	151
<b>Figure 5.ix.</b> Reduction in Hs68 cell-associated IGFBPs mediated by TNF $\alpha$ .....	152
<b>Figure 6.i.</b> Western ligand blot of Hs68 cell IGFBPs following 24 hour treatment with IL-1 $\beta$ and IL-6.....	159
<b>Figure 6.ii.</b> Dose-dependent IL-1 $\beta$ inhibition of immunoreactive IGFBP-3 levels in Hs68 cell conditioned medium.....	161
<b>Figure 6.iii.</b> The effect of IL-1 $\beta$ on the inhibition of Hs68 cell IGFBP-3 by TNF $\alpha$ .....	162
<b>Figure 6.iv.</b> Dose-dependent IL-1 $\alpha$ inhibition of immunoreactive IGFBP-3 levels in Hs68 cell conditioned medium.....	163
<b>Figure 6.v.</b> Northern blot of Hs68 cell IGFBP-3 and -4 mRNA following treatment with IL-1 $\beta$ and IL-6.....	164
<b>Figure 6.vi.</b> The effect of IL-6 upon immunoreactive IGFBP-3 levels in Hs68 conditioned medium.....	166
<b>Figure 7.i.</b> Western ligand blot of Hs68 cell IGFBPs following 24 hour treatment with TGF $\beta$ <sub>1</sub> .....	173
<b>Figure 7.ii.</b> Dose-dependent TGF $\beta$ <sub>1</sub> stimulation of immunoreactive IGFBP-3 levels in Hs68 cell conditioned medium.....	174
<b>Figure 7.iii.</b> The effect of cell density upon TGF $\beta$ <sub>1</sub> stimulation of IGFBP-3 in Hs68 cells.....	175
<b>Figure 7.iv.</b> Fibronectin involvement in the IGFBP-3 response to TGF $\beta$ <sub>1</sub> in Hs68 cells.....	177
<b>Figure 7.v.</b> Northern blot of Hs68 cell IGFBP-3 and -4 mRNA following treatment with TGF $\beta$ <sub>1</sub> .....	178
<b>Figure 7.vi.</b> The effect of 24 hour TGF $\beta$ <sub>1</sub> treatment upon Hs68 cell IGFBP-3 protease activity.....	180
<b>Figure 8.i.</b> Comparison of IGF-I bioactivity in Hs68 cells	



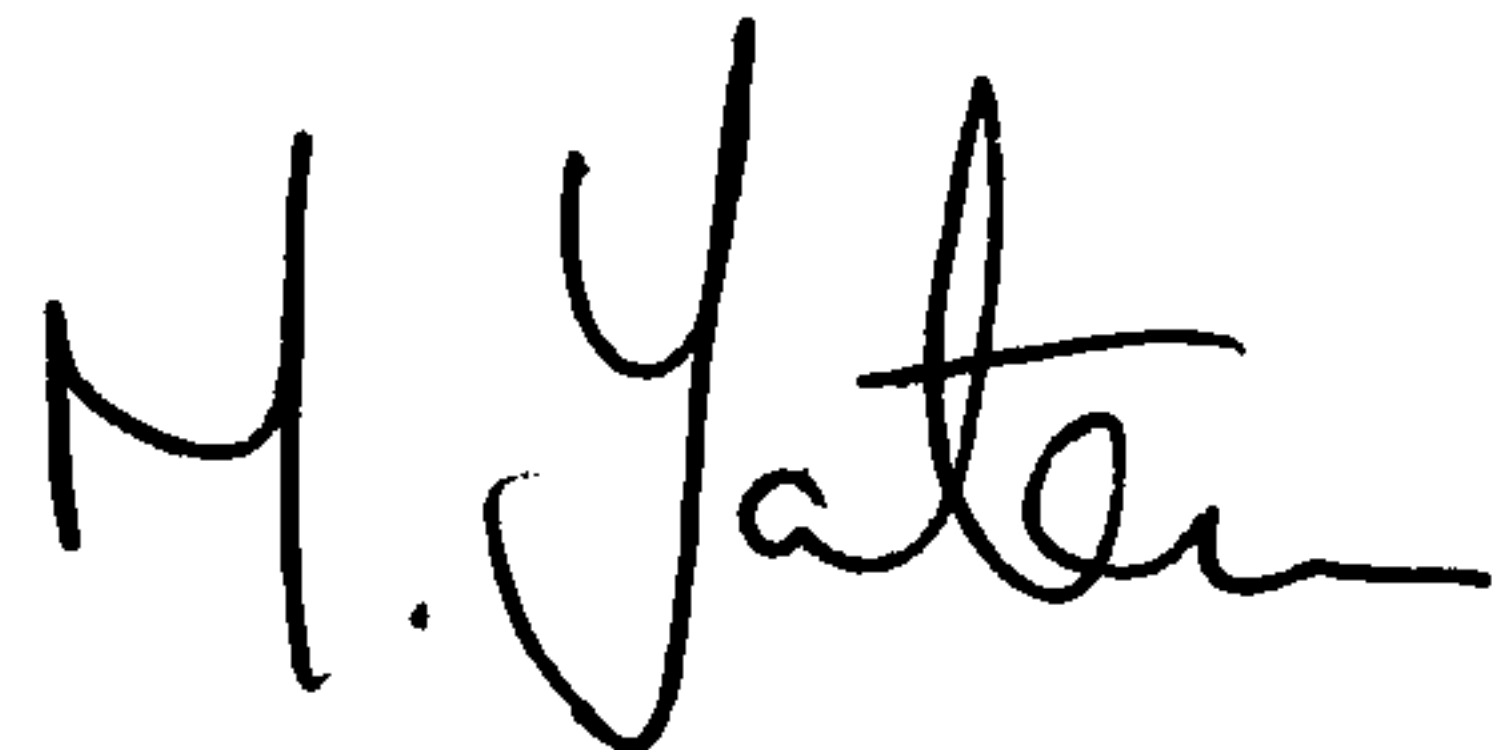
when assessed by cell counting or MTT bioassay.....	186
<b>Figure 8.ii.</b> Dose-dependent IGF-I stimulation of Hs68 cell proliferation assessed by the MTT bioassay.....	187
<b>Figure 8.iii.</b> The effect of co-incubating exogenous IGFBP-3 upon the bioactivity of IGF-I and LongR3-IGF-I.....	188
<b>Figure 8.iv.</b> The effect of pre-incubating exogenous IGFBP-3 upon the bioactivity of IGF-I and LongR3-IGF-I.....	190
<b>Figure 8.v.</b> Dose-dependent stimulation of Hs68 cell proliferation by TNF $\alpha$ and TGF $\beta_1$ .....	191
<b>Figure 8.vi.</b> The effect upon Hs68 cell proliferation of co-incubating TNF $\alpha$ and IGF-I.....	193
<b>Figure 8.vii.</b> The effect upon Hs68 cell proliferation of co-incubating TNF $\alpha$ and LongR3-IGF-I.....	194
<b>Figure 8.viii.</b> The effect upon Hs68 cell proliferation of co-incubating TGF $\beta_1$ and IGF-I.....	196
<b>Figure 8.ix.</b> The effect upon Hs68 cell proliferation of co-incubating TGF $\beta_1$ and LongR3-IGF-I.....	197

## LIST OF TABLES

<b>Table 1.</b> Historical and current nomenclature of IGF peptides.....	17
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## ATTRIBUTION

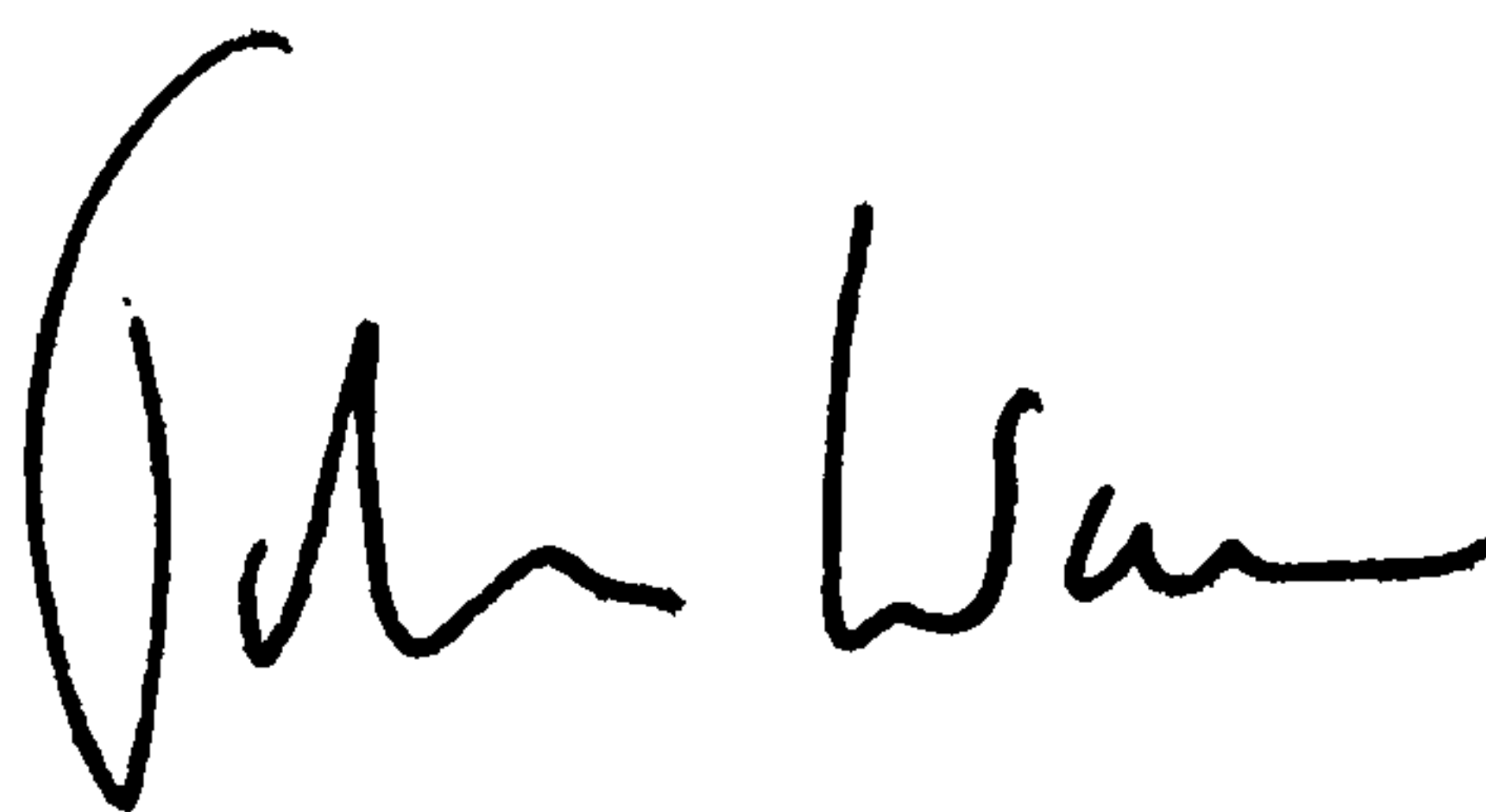
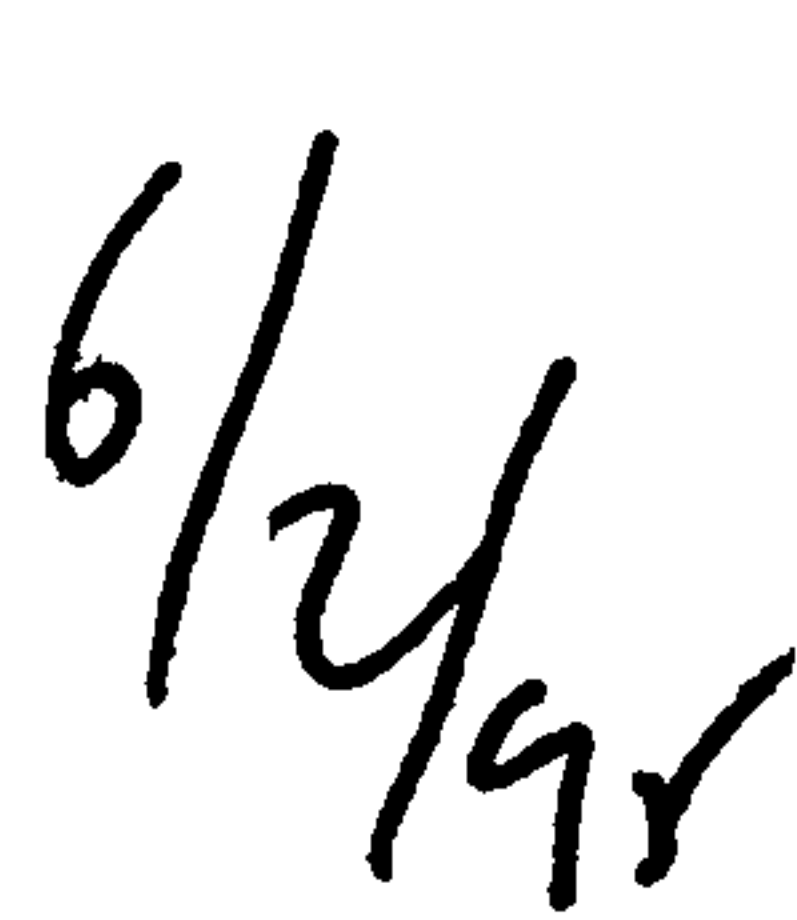
I declare that this thesis has been composed by myself, and that the work of which it is a record has been principally performed by myself. The statistical analysis of much of the data within this thesis was performed under the invaluable direction of Dr Nigel Yateman.



Signed

Martin E. Yateman

I declare that the conditions of the ordinance and regulations (Ph.D) have been fulfilled.

Signed

Professor John A.H. Wass  
Supervisor.

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Finally, I should like to pay utmost gratitude to my wife Bridget for all her support, encouragement and motivation.

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## **DEDICATION**

I dedicate this thesis to my wife Bridget, and also to the institution of St.Bartholomew's Hospital.

## ABBREVIATIONS

APS	Ammonium persulphate
ALS	Acid labile subunit
bp	Base pairs
BSA	Bovine serum albumin
cDNA	Complementary DNA
CM	Conditioned medium
DEPC	Diethylpolycarbonate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra-acetic acid
FCS	Fetal calf serum
FPLC	Fast phase liquid chromatography
GH	Growth hormone
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
<sup>125</sup> I	Radioactive isotope of iodine
IGF-I	Insulin-like growth factor I
IGF-II	Insulin-like growth factor II
IGFBP	Insulin-like growth factor binding protein
IFN-g	Interferon gamma
IL-1	Interleukin 1
IL-6	Interleukin 6
kb	Kilo base
kDa	Kilo daltons
MEM	Minimum essential medium
mRNA	Messenger RNA
MTT	3-[4,5-dimethyl thiazol-2]-2,4-diphenyl tetrazolium bromide

$^{32}\text{P}$	Radioactive isotope of phosphorus
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
$\text{PGE}_2$	Prostaglandin $\text{E}_2$
RIA	Radioimmunoassay
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SFM	Serum-free medium
TEMED	N,N,N,N,-tetramethylethylenediamine
$\text{TGF}\beta$	Transforming growth factor $\beta$
$\text{TNF}\alpha$	Tumour necrosis factor $\alpha$
v/v	volume to volume
WLB	Western ligand blot
w/v	weight to volume



**CHAPTER 1.**  
**INTRODUCTION**

## **CHAPTER 1.**

### **SECTION A. IGF CHARACTERISATION**

#### **1.1. Historical perspectives**

The first evidence for a secondary mediator of growth hormone (GH) action came in 1957 from Salmon and Daughaday, with their discovery that while GH *in vivo* stimulated the incorporation of sulphate into cartilage, it was inactive *in vitro*. They then presented evidence for an active factor in serum, under the control of GH, that stimulated *in vitro* sulphate incorporation into chondroitin sulphate in rat costal cartilage segments. Accordingly they named this mediator of GH action 'sulphation factor'.

Further research demonstrated that 'sulphation factor' also stimulated synthesis in chondrocytes of DNA, RNA, protein and hydroxyproline (Daughaday and Reeder, 1966), and promoted the proliferation of cultured cells in serum-free media. In recognition of these properties, the limiting term 'sulphation factor' was replaced by the name somatomedin (Sm) to reflect what was thought to be its primary function, the mediation of the growth promoting effect of GH (Salmon and DuVall, 1970). Three somatomedins were originally described, somatomedin-A (Sm-A) being defined by classical sulphation activity in chick cartilage (Sievertsson *et al*, 1975), somatomedin-B (Sm-B) by its ability to stimulate DNA synthesis in glial cells (Uthne, 1973), and somatomedin-C (Sm-C) by sulphation activity in a rat cartilage assay (Van Wyk *et al*, 1974). However, subsequent amino acid sequence analysis has since shown that Sm-A was a deaminated form of Sm-C (Enberg *et al*, 1984), whilst the original Sm-B preparation was found to be contaminated with epidermal growth factor (Heldin *et al*, 1981).

At a similar time to the discovery of the somatomedins, another group was studying the nature of the insulin-like activity in serum detected in bioassay but absent in an insulin immunoassay (Froesch *et al*, 1963). The addition of saturating amounts of insulin antibodies to serum failed to affect the insulin-like bioactivity, namely glucose uptake, by more than 10%. The remaining 90% was thus termed the non-suppressible insulin-like activity (NSILA), and was found to consist of a low molecular weight acid-ethanol soluble fraction (NSILA-s) and a high molecular weight precipitable fraction (NSILA-p) (Froesch



1967). This seems to be the first description of the binding protein complex. The NSILA-s of approximately 8000 kDa was later found to possess similar properties to the somatomedins; stimulating sulphate incorporation into cartilage (Zingg and Froesch 1973), and DNA synthesis in fibroblasts (Morell and Froesch 1973, Zapf *et al*, 1978).

As evidence for somatomedins and NSILA was emerging, another circulating peptide that also had the ability to stimulate DNA synthesis in cultured chicken embryo fibroblasts was partially purified from calf serum (Pierson and Temin, 1972). They named this somatomedin-like peptide multiplication-stimulating activity or MSA, and later showed it to be secreted by the rat liver cell line designated BRL-3A (Dulak and Temin, 1973). Confusion surrounding these early descriptions was finally resolved following analysis of the primary structure of the somatomedins, NSILA and MSA showing the activity of all these peptides could be ascribed to one of two factors which were then designated insulin-like growth factor (IGF) -I or IGF-II, Daughaday *et al* (1987). This remains the present day nomenclature, see **Table 1**.

**Table 1.**

<u>ORIGINAL DESCRIPTION</u>	<u>PRESENT CLASSIFICATION</u>
SULPHATION FACTOR SOMATOMEDIN-C SOMATOMEDIN-A NSILA	IGF-I
NSILA MSA	IGF-II

**1.2. IGF peptide structure**

The primary structures of IGF-I and IGF-II were first characterised from adult human plasma by Rindernecht and Humbel (1978, 1978a). They are both single chain peptides



with three interchain disulphide bridges and are comprised of either 70 (IGF-I) or 67 (IGF-II) amino acids, with molecular weights of 7646 and 7471 Daltons, respectively. The IGF structures functionally consist of an A-domain and an amino terminal B-domain separated by a connecting C-region (**figure 1.i.**). IGF-I and IGF-II share identical amino acids at 45 positions, a 64% homology, whilst the A and B regions show a 42% sequence homology with proinsulin. Unlike proinsulin however, the IGFs have an additional D-domain which extends from from the C-terminal end of the peptide.

The primary structure of the IGFs has also been characterised in a number of other species, including rat (Murphy *et al*, 1987), mouse (Bell *et al*, 1986), cow (Honneger & Humbel, 1986) and pig (Tavakkol *et al*, 1988). Human, bovine and porcine IGF-I were seen to be identical demonstrating the highly conserved nature of the peptide. Rat and mouse IGF-I differ from the human form by only three and four amino acids, respectively. Sequence analysis of isolated IGF cDNA clones has predicted that both IGF-I and IGF-II are initially synthesized as large 18 kDa precursors, with additional E-domain regions at the carboxy termini (**figure 1.i and figure 1.ii.**). IGF-I can have a 35 (IGF-IA) or 77 (IGF-IB) amino acid extension, while IGF-II exists with an extra 89 amino acids.

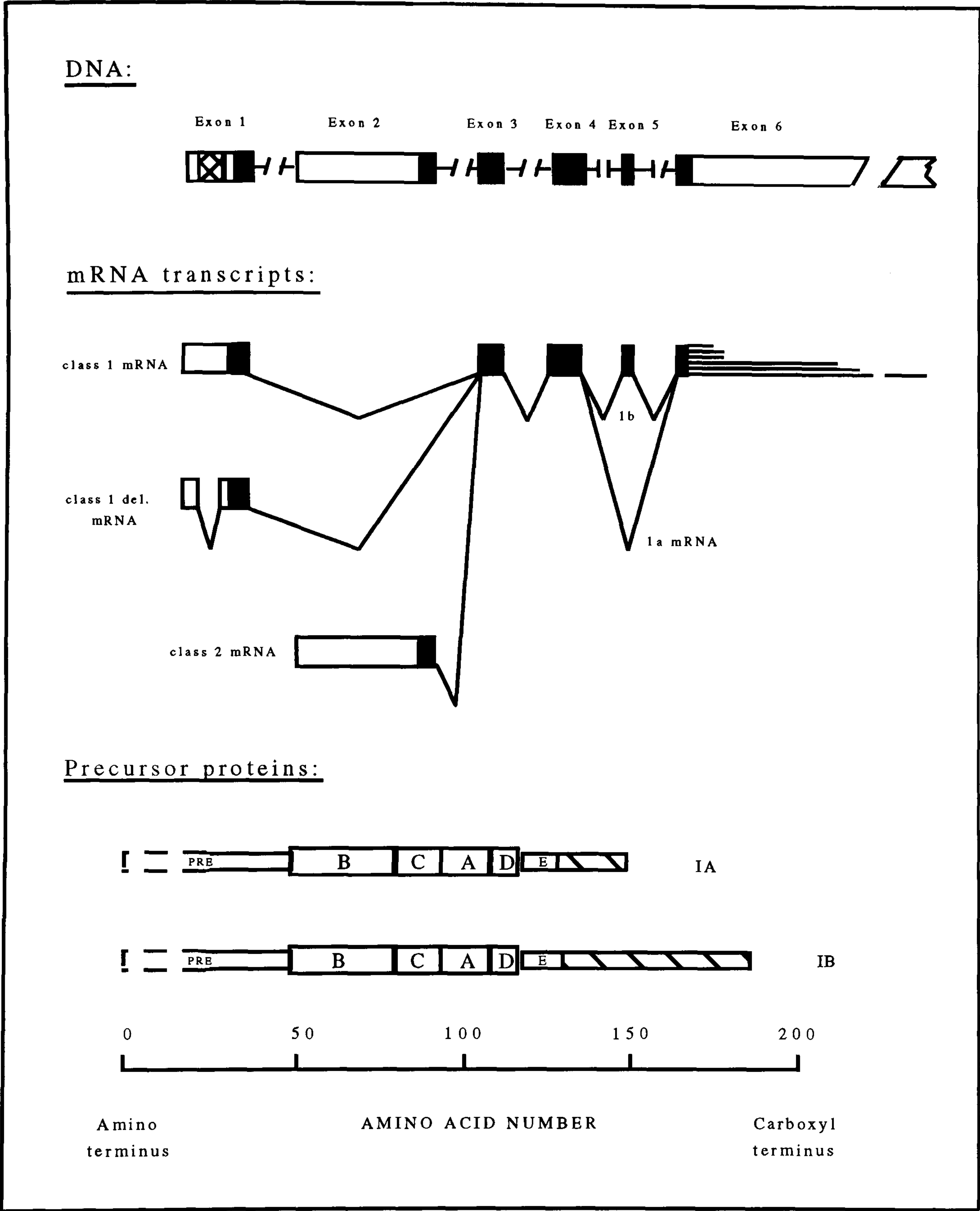
Certain other altered forms of IGFs have also been described. A truncated IGF-I peptide named Des[1-3]-IGF-I, with a deletion of the first three amino acids at the N-terminus, has been isolated from both human brain (Sara *et al*, 1986) and bovine colostrum (Francis *et al*, 1988). This IGF-I peptide whilst having normal affinity for the type 1 IGF receptor, has a greatly reduced affinity for the IGFBPs.

A number of circulating precursor IGF-II peptides have also been observed. For example, Daughaday *et al* (1988) reported a 15 kDa form in normal plasma and also in patients with non-islet cell tumour associated hypoglycemia. Its biological activity was similar to the mature 7.5 kDa form. A 10 kDa IGF-II peptide, with a 21 amino acid extension, has also been purified from human serum (Zumstein *et al*, 1985).

### **1.3. Gene structure**

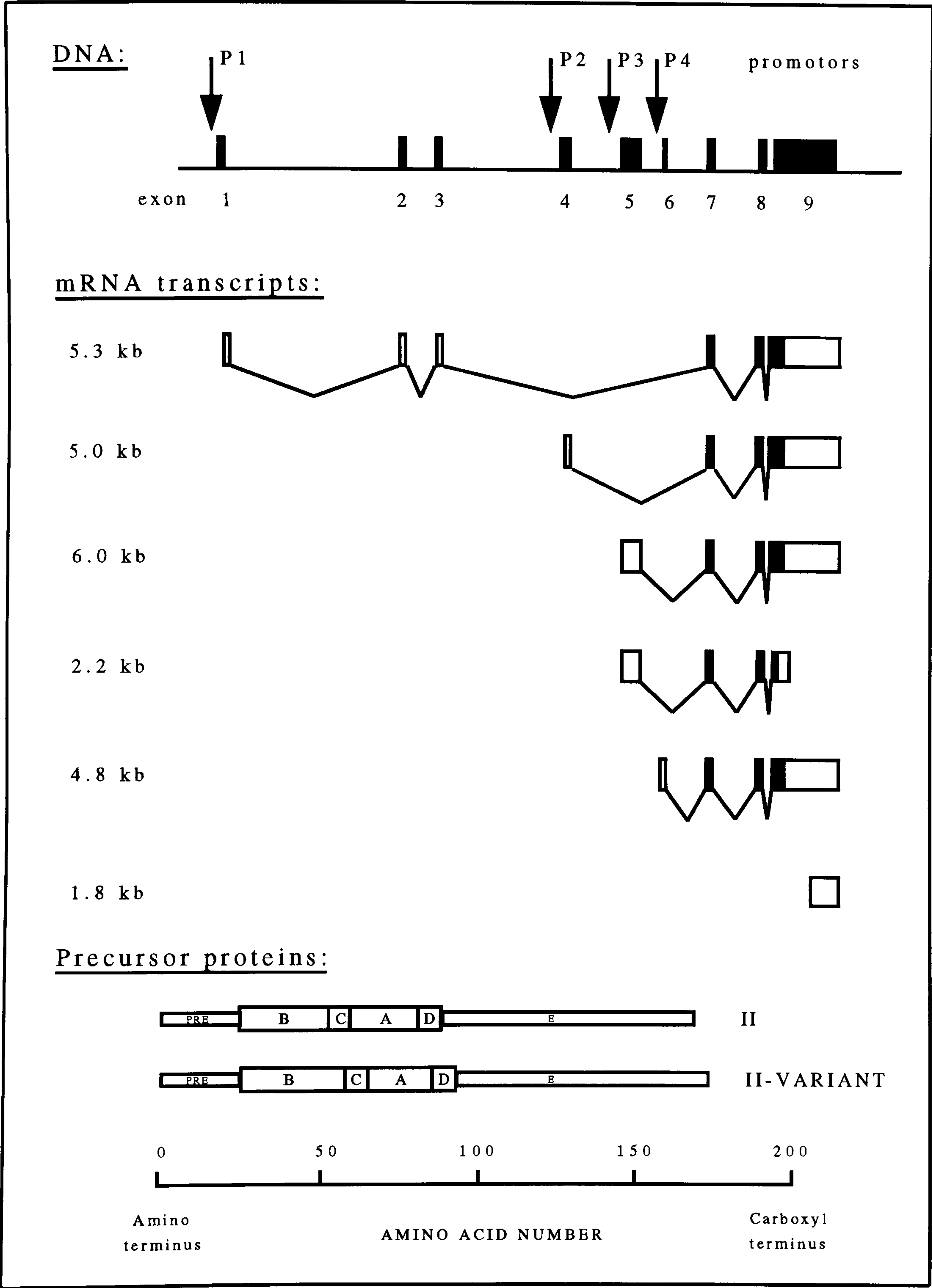
The genomic sequences for IGF-I and IGF-II have been mapped to the long arm of human chromosome 12 and the short arm of chromosome 11 respectively (Brissendon *et al*, 1984)

Figure 1.i.



Schematic representation of mammalian IGF-I DNA, mRNA transcripts and precursor proteins. Adapted from Holthuisen et al, 1991.

Figure 1.ii.



Schematic representation of mammalian IGF-II DNA, mRNA transcripts and precursor proteins. Adapted from Holthuizen et al, 1991.



Recent molecular cloning studies have indicated that in mammals the single-copy IGF-I gene has both a complex structure and a complicated pattern of expression.

In rats and humans there are six IGF-I exons and at least four promoters distributed over more than 70 kb of chromosomal DNA. The first two leader exons at the 5'-part of the human IGF-I gene, designated 1B and 1C, contain alternative initiation sites of transcription. The mature IGF-I peptide is encoded by exons 2 and 3. The variable E domains seen in the IGF-I precursor peptides arise from alternative splicing of the class I mRNA; exon 3 to 4 in IGF-IA and exon 3 to 5 in IGF-IB. Northern blot analysis has subsequently shown that exon 4 is only present in the 1.3 kb mRNA, while exon 5 is found in the 7.6 and 1.1 kb mRNA species.

Each of these mRNAs encode IGF-I precursors with different carboxyl-terminal extensions, perhaps giving a subtle range of different biological activities. Moreover, there are indications in the rat that the shorter mRNA species are preferentially associated with polysomes and have a higher stability. The 7.6 kb mRNA also has several putative destabilisation signals, thought to be important in determining its half-life.

The 30 kb IGF-II gene is similarly complex, with nine exons and four different promoters generating five or more mRNA species, ranging from 2.2 - 6.0 kb in size (**figure 1.ii.**). It is also notable that the IGF-II gene is located very close to the insulin gene, being separated by only 1.4 kb of intergenic cDNA.

#### **1.4. IGF function**

Despite the wide range of activities that have been described for the IGFs, most fall into three broad categories: those affecting mitogenesis, insulin-like metabolic actions and those involved in modulating cellular differentiation.

##### **1.4.i. Mitogenic effects**

Perhaps the most widely acknowledged function of the IGFs is to stimulate cell proliferation. This ability of IGF was first demonstrated *in vitro* by Salmon and Hosse (1971) who showed that a serum fraction rich in 'sulphation activity' could partially





substitute for serum in stimulating the growth of HeLa cell populations. The partially purified NSILA and MSA (Pierson and Temin, 1972) was then shown to stimulate the incorporation of  $^3\text{[H]}$ thymidine into cellular DNA *in vitro*, a now well established indicator of mitogenesis.

Demonstration of the ubiquitous nature of the mitogenic IGF response has subsequently come from a large number of studies measuring thymidine uptake in a variety of cell types. These include foreskin fibroblasts (DeMellow and Baxter, 1988), thyroid epithelial cells (Tramontano *et al*, 1986), myoblasts (Florini *et al*, 1986), and hepatoma cells (Verspohl *et al*, 1988). Despite such widespread target cell types however, the IGFs only appear to be effective at certain points in the cell cycle. For example, cells that have become quiescent and have entered into  $G_0$  of the cycle, usually through the removal of the serum component in the culture medium, are often insensitive to the mitogenic action of the IGFs. The addition of 'competence factors' are often required, such as platelet derived growth factor (PDGF) and fibroblast growth factor (FGF), which initiate the cell cycle and take the cells into  $G_1$ . Thus stimulated, the IGFs can then act as 'progression factors' taking the cells into the DNA synthesis stage (S) and then through stages  $G_2$  and M, ending in cell division (Clemmons and Van Wyk *et al*, 1981).

The wide ranging mitogenic effects of the IGFs seen *in vitro* are thought to be seen *in vivo* translating GH signals into effects on normal body growth. IGF-I for instance stimulates the clonal expansion of prechondrocytes in the germinal layer of the epiphyseal growth plate, which after degenerating allows increased cartilage matrix to be ossified by osteoblasts, resulting in new bone formation. Close correlations between the GH / IGF axis and body height scores during development from neonate to adult have been well documented, with increased circulating IGF-I levels seen during the pubertal growth spurt. Moreover, abnormalities of growth such as those caused by GH excess (acromegaly) and GH insensitivity (Laron) syndrome are characterised by correspondingly altered IGF-I levels (Clemmons *et al*, 1979, Laron *et al*, 1980).



#### **1.4.ii. Insulin-like metabolic effects**

Early studies using the so named 'fat pad assay' (Froesch *et al*, 1963) described the ability of IGF to stimulate glucose uptake and net gas exchange in rat epididymal fat pad sections. Purified IGF-I and IGF-II were shown to be approximately sixty-fold less potent than insulin in this assay (Zapf *et al*, 1978), although the potential for IGF controlled glucose regulation would appear great due to its vastly higher concentration in serum. Similarly, the IGFs were shown to stimulate  $^{14}\text{CO}_2$  production and  $^{14}\text{C}$  incorporation into lipid (lipogenesis) in isolated fat cells, with IGF-II two to three-fold more potent than IGF-I.

A number of other IGF bioassays have also employed the use of radiolabelled metabolites as indices of metabolic activity. These include IGF stimulated cellular uptake of D-[1- $^3\text{H}$ ]glucose and its incorporation into glycogen, the uptake of the non-metabolised 2-deoxy-D-[1- $^3\text{H}$ ]glucose, the incorporation of L-[4,5- $^3\text{H}$ ]leucine into Sertoli cell protein (Borland *et al*, 1984), and the uptake of the non-metabolised 2-amino[1- $^{14}\text{C}$ ]isobutyric acid by human fibroblasts (Kaplowitz, 1987) and rat thyroid cells (Rotella *et al*, 1989). Despite early suggestions that such effects were due to IGF cross-reacting with the insulin receptor, many of the above actions can be assigned to the IGF receptors themselves, although the overall IGF contribution to the acute regulation of glucose metabolism *in vivo* is unclear at present.

Certain metabolic enzymatic activities can also be regulated by the IGFs, such as increased glucose-6-phosphate dehydrogenase activity in human osteosarcoma cells (Farquharson *et al*, 1992), and increased glycogen synthase activity in isolated soleus muscle (Poggi *et al*, 1979).

#### **1.4.iii. Cellular differentiation**

The IGFs have been demonstrated to stimulate the differentiation of myoblasts (Florini *et al*, 1986), osteoblasts (Schmid *et al*, 1984), and adipocytes (Smith, 1988). They also appear to be differentiating factors for both ovarian (Adashi *et al*, 1985) and testicular function (Chatelain *et al*, 1987), amplifying the actions of the respective steroid hormones. The IGFs can induce erythropoiesis (Claustres *et al*, 1987) and also stimulate



granulopoiesis and chemotaxis in endothelial and melanoma cells (Stracke *et al*, 1988). Differentiated effects under IGF control also include the stimulation of sulphate uptake into costal cartilage and the synthesis and deposition of glycosaminoglycans into the extracellular matrix (Kemp and Hintz, 1980).

### **1.5. Control of IGF secretion**

Historically, studies investigating the regulation of IGF activity have tended to follow the same route used for other established peptide hormones, largely focussing therefore on the alteration of IGF gene expression and peptide levels in the circulation. A number of factors and conditions which are capable of mediating these parameters have now been identified and are described below.

Daughaday *et al* (1976) first demonstrated that IGF-I secretion from isolated rat liver, since recognised as the main source of circulating IGF-I, was particularly sensitive to GH. Since this initial discovery, GH has been established as the primary regulator of IGF-I gene expression (Mathews *et al*, 1986), not only of hepatic origin but also in many other tissues, such as heart, lung and pancreas.

The GH dependency of circulating IGF-I levels is also evident in a number of clinical conditions involving altered GH status, such as puberty, acromegaly and hypopituitarism, whilst the lack of functional GH receptors in fetal tissues and Laron syndrome has been suggested to be responsible for their low IGF-I levels. Unlike the pulsatile nature of GH secretion however, IGF-I levels do not undergo any marked diurnal rhythm.

There is also evidence to suggest other trophic hormones can regulate IGF biosynthesis, particularly that of extrahepatic origin. For example, increased IGF-I gene expression can result from treatment with platelet derived growth factor (PDGF) and fibroblast growth factor (FGF) in human fibroblast cultures (Clemmons & Shaw, 1983), thyroid hormones in the liver (Tollet *et al*, 1990), epidermal growth factor in the kidney (Rogers *et al*, 1991), and parathyroid hormone in cultured foetal rat bone cells (McCarthy *et al*, 1989). The effects of these hormones do appear to be tissue specific however, as demonstrated by oestradiol stimulating IGF-I in the uterus (Murphy and Friesen, 1988), whilst generally



inhibiting the hepatic IGF output. Follicle stimulating hormone (FSH), luteinising hormone (LH), and adrenocorticotrophic hormone (ACTH) act on their respective target organs to stimulate paracrine production of IGF-I.

Nutrition is also an important regulator of circulating IGFs, with severe fasting, such as that seen in patients with anorexia nervosa, reducing IGF-I levels to those associated with GH deficiency (Clemmons *et al*, 1981). In these states, GH is rendered ineffective and only refeeding can restore IGF-I levels to normal.

An enhancement of IGF-I levels is seen at sites of local tissue injury. An elevation in IGF-I immunoreactivity during regeneration after injury has been observed in rat peripheral nerves, skeletal muscle, and endothelial cells of arteries (Jennische *et al*, 1987). Although the precise mechanism behind such an effect is unclear, a possible source of IGF other than from an autocrine origin may be from invading cells involved in the inflammatory response, such as platelets and peripheral blood mononucleocytes.

## **1.6. Cell membrane IGF receptors**

Another area of IGF physiology which is crucial to the regulation of the activity of this growth factor is the nature and tissue distribution of the cell membrane IGF receptor family. Like most peptide hormones and growth factors the IGFs require association with one of a group of cell surface receptors to elicit their biological actions. Early competitive binding experiments demonstrated the presence of two subtypes of IGF receptors quite distinct from the insulin receptor and have subsequently been designated as Type I and Type II IGF receptors.

### **1.6.i. Type I IGF receptors**

Type I IGF receptors were characterised by Ulrich *et al* (1986) and are closely related to the insulin receptor, with about a 40% homology. They are initially synthesised as 150 kDa precursor proteins, which are subsequently glycosylated, proteolytically cleaved and dimerized to form the 300 - 350 kDa heterotetrameric receptor structure. This consists of two extracellular  $\alpha$  subunits (135 kDa) anchored to the plasma membrane and two



transmembrane  $\beta$  subunits by disulphide bonds.

The  $\beta$  subunits (90 kDa) contain an intracellular portion with an adenosine triphosphate (ATP) binding region, autophosphorylation sites and intrinsic tyrosine-specific protein kinase activity (Roth *et al*, 1991). Although the precise biochemical cascade of events that follows activation of these tyrosine kinases upon IGF receptor ligand interaction is largely unknown, certain phosphatidylinositol kinases (type I PtdIns kinase) appear to be closely involved in a similar manner to insulin.

Unlike the rather ubiquitous insulin receptor, Type I IGF-I receptors can exist in varying quantities on cell surfaces or be present in specific cell type subpopulations. Scatchard analysis of IGF-I binding data has demonstrated the presence of such receptors on normal human fibroblasts (Rosenfeld and Dollar, 1982) with a high affinity for the IGF-I peptide ( $K_a$  of  $1.07 \times 10^9 \text{ M}^{-1}$ ) similar to that seen in IM-9 human lymphocytes and BRL 3A2 rat liver cells. They also strongly bind IGF-II with a  $K_a$  approximately ten-fold reduced (Ritvos *et al*, 1988) and to a lesser degree insulin.

#### **1.6.ii. Hybrid IGF-I receptors**

The characteristic modular structure of the Type I IGF receptor and its similarity with the insulin receptor can apparently give rise to the existence of hybrids. Essentially, these consist of one pair of  $\alpha$  and  $\beta$  subunits from each of the respective receptors, thus retaining the tetrameric tertiary structure (Soos and Siddle, 1989), and have been found in various tissues, including hepatoma and fibroblast cells.

Their binding affinity appears greater for IGF-I than insulin, although it is not known which of the tyrosine kinase domains within the hybrids becomes activated when either ligand binds. As the IGF and insulin components of these receptors are independently regulated however it seems likely that they may serve to increase the flexibility of cellular responses to certain extracellular stimuli.

#### **1.6.iii. Type II IGF receptors**

The primary structure of the Type II IGF receptor was first characterised in human hepatoma (HepG2) cells by Morgan *et al* (1987). These single-chain 220-300 kDa



receptors were thus found to be structurally unrelated to both type I IGF and insulin receptors, with high binding affinity for IGF-II ( $K_d$  of 0.1 nM, Beukers *et al*, 1991) and little or no affinity for either IGF-I or insulin.

They consist of a large extracellular domain, which accounts for approximately 93% of the total receptor protein, comprising fifteen hydrophobic cysteine-rich repeat sequences. This is linked by a single transmembrane region to a small cytoplasmic domain, which unlike the Type I IGF receptor has no protein kinase activity, although can be phosphorylated in intact cells.

More recent evidence has suggested that secondary signalling by IGF-II through this receptor may involve the activation of certain calcium channels mediated via a network of pertussis toxin-sensitive G proteins, such as  $G_{i-2}$  (Nishimoto *et al*, 1989).

Perhaps the most intriguing discovery about the Type II IGF receptor came however from its cloning analysis which showed it to be identical to the cation independent mannose-6-phosphate (Man-6P) receptor (Oshima *et al*, 1988). Ligand binding studies demonstrated that each receptor could bind both IGF-II and molecules containing the Man-6P residue without inhibiting the binding of each other, indicating separate binding sites. Moreover, as the Man-6P receptor has been implicated in targeting molecular traffic to cytoplasmic lysosomes for degradation, possible IGF-II modulation of such a process, although as yet unproven, remains a possibility.

#### **1.6.iv. IGF receptor regulation**

The dynamic nature of cell membrane receptor populations, such as those for the IGFs, means that their regulation can take a number of forms. Essentially, regulation of receptor activity may be achieved by alteration of receptor affinity or number, the latter reflecting changes in receptor biosynthesis (principally gene expression), degradative processing and internalisation. Altered Type I IGF receptor gene expression has been recorded in response to a variety of conditions. Nutritional deprivation, for example, can cause reversible increases in steady state mRNA and receptor number in lung, stomach, kidney and heart tissues (Lowe *et al*, 1989). Developmental stage also determines the mRNA levels of this receptor, with a general reduction seen in many rat tissues following embryonic



advancement and postnatal development.

Certain hormonal regulators have also been observed to alter Type I IGF receptor gene expression. Estradiol increases mRNA levels 6.5 fold in a human breast cancer cells (MCF-7), whilst there is some evidence that diabetes can lead to significant increases in some rat tissues.

Down regulation of Type I IGF receptor numbers by IGF-I, IGF-II and insulin, in proportion to their relative binding affinities, has been observed *in vitro* using human lymphocytes and fibroblasts (Rosenfeld and Dollar, 1982). This effect is not however seen in Type II IGF receptors, once again reflecting their markedly different nature. Indeed, insulin appears to acutely upregulate Type II receptors in rat adipocyte tissue, the most likely mechanism being an alteration in its rapid turnover and recycling, possibly by inhibiting kinase activity (Corvera and Czech, 1985). GH similarly affects adipocyte Type II receptors (Lönnroth *et al*, 1987).

#### **1.6.v. Functional aspects of IGF receptors**

Initially, largely due to the ease at which IGFs cross-reacted with the insulin receptor, it was assumed that the IGFs mediated their insulin-like metabolic actions through this receptor. However, a number of studies have now shown that whilst most of the growth-promoting action of the IGFs (and insulin) are mediated via the Type I IGF receptor, many metabolic IGF actions also result from the interaction with the receptor in a number of tissue types. For example, IGF metabolic activity such as glucose and amino acid uptake has been recorded to occur through Type I IGF receptors in human skin fibroblasts (Knight *et al*, 1981), human choriocarcinoma cells (Ritvos *et al*, 1988a), human hepatoma cells (Vershpohl *et al*, 1988) and even mouse muscle (Poggi *et al*, 1979).

The role of the Type II IGF receptors is more unclear however. In many cell types IGF-II mitogenesis can be inhibited by the addition of *Type I* IGF receptor monoclonal antibodies (Conover *et al*, 1986), whilst anti-Type I IGF receptor antibodies have mostly been ineffective. In a few tissues, such as the human erythroleukaemic cell line K562 which lacks the Type I receptors, IGF-II can stimulate cell growth via its own receptor however (Tally and Hall, 1990), indicating that this receptor can transmit well recognised IGF

signalling functions, in some cells at least. As outlined earlier, the significance of the Man-6P receptor homology is unknown at present.



## **CHAPTER 1.**

### **SECTION B. THE INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS**

The most unique aspect which characterises IGF physiology compared to other peptide hormones and growth factors is the existence of a closely related family of soluble binding proteins. The six species of IGFBPs so far identified seem to share a common ancestry, as demonstrated by the high degree of homology in their amino acid sequences (**figure 1.iii.**), notably in that part of the molecule thought to be important in IGF binding, the N and C termini. Despite such close structural similarities the IGFBPs have variable IGF binding affinities, often greater or of the same magnitude as the cell membrane IGF receptors, and unlike GH binding protein they are not simply soluble components of the membrane receptors but arise from distinct genes. It has also become increasingly apparent that individual IGFBP species are often localised to particular tissues or cell types and may be regulated by their own specific factors, suggesting that they are not merely simple carrier proteins but may have a more active role in determining IGF physiology. An overview of the current knowledge on the IGFBPs is now given below.

#### **1.7. Insulin-like growth factor-binding protein 3**

##### **Characterisation**

The majority of circulating IGFs in man, especially IGF-I, are found in a ternary complex of approximately 140 kDa. This comprises an acid-labile ( $\alpha$ ) subunit of about 85 kDa, the IGFBP-3 acid stable binding ( $\beta$ ) subunit of 50 kDa and the growth factor subunit of about 7.5 kDa, either IGF-I or IGF-II (Furlanetto, 1980). The  $\alpha$ -subunit appears on non-reduced SDS-PAGE as a glycoprotein doublet between 84-86 kDa, with binding to the IGFBP-3 only possible when the IGFBP-3 is occupied by the IGF peptide (Baxter, 1988).

The  $\beta$ -subunit of this complex, the IGFBP-3 peptide, also appears on non-reduced SDS PAGE as a doublet of approximately 53 and 47 kDa, both with IGF binding activity. These two bands represent differentially glycosylated forms of the same 29 kDa core peptide which has been cloned and sequenced (Wood *et al*, 1988). The complete cDNA for human IGFBP-3 is approximately 2500 bp in length, containing a 120 bp 5'



1

APWQCaPCsAEkLALcPvSAS-----CSE-----vTRsAGCGCCpMcalPLgAAcGVATaRCARGLsCrALPGEqqPLHALtRGqGAC...

2

EVlFRCPpCTpErLAaCGppvVAPPAAVAAGGArMpCaE----LVREPGCGCCsvCARLEGEACGVYTPRCGqGLRCyHPGsELPLqALvmGEgTC...

3

GASSGGLGPvVRCEPCDaRALAQCaPPPAV-----CaE-----LVREPGCGCCLTcALsEGQpCGIYTERCGsGLRCpSPDEaRPLqALLdGRGLC...

4

DEAIHCPPcSEELARcRPPVG-----CE-----LVREPGCGCCATcALgLGMPcGVYTPRCGsGLRCyPpRGvEkPLHtLMHGqGvC...

5

LGSFVHCePCDEKALsmC-PPsPLG-----C-E-----LVkEPGCGCCmTcALaEGQsCGVYT\_ERCaQGLRC\_LPrQDEEkPLHALLHGRGvC...

6

ALARCPgCGQGVQAGC-PGG-----CvEEEDGGSPAEgCAEAEGCLRRREcQ\_ECGVYTP\_NCaPGLqChP\_PkDdEaPLrALLLGRGrC...

...

(VQESDASAPHAaEAGSPeSTEITEEELDNFHLMAPSEEDHSILWDAISTYDGSKALHVTNIKKWKE)...

...

(EKRRDAEYGASPEQVADNGDDHSEGGLVENHVDSTMNMLGGGSGAGRKPLKSGMKELAVFREKvTEQHRQMGGKHHLGLEEPKKLRPPPPART)...

...

(VNASAVSRLRAYLLPAPPAPGNASEEEDRSAGSVEsPSvSSThRVSDPKFHPLHSKI\_I\_IKKGHAKDSQRyKVdYESQsTDtQNFSSeSKRETEYG)...

...

(MELAEIEAIQESLQPSDKDEGDHPNNSFSPCSahDRRClQKHfAKIRDRSTSGGKMkVNGAPREDARpVPVpQG)...

...

(LNEKSYREQVKIERDSREHEEPTTSEMAEETySPKIfrPKHTRISELKAeAVKKDRKKLTQSKfVGGAEntaHPRIISaPEMRQeSEQG)...

...

(LPARAPAVAEENPKESKPQAGTARPDVNRRDQQRNPGTSTTPSQPNsAGVQDTEMG)...

...

PCRiELyRvEsLAKAQETs--GE-E-ISKfYLpNCnKnGFYHSRQCETsMDGEaGLCwCVYPwNGKRIpGsPeIRGDpNCQMYFNvQN

...

PCqQELDqVLErIStMRLPDERGpLEhLYslHIPNCdKHGLYNLkQCkMSLnGqRGEcWCVNPNTGKLIqGAPtIRGDPeChLFYNEQQEaCGvHTQRMQ

...

PCRREMEDTLNHl\_KfLNVLsPRGV-----HIPNCdKkGFYkKkQCQRPSkGRkRGfCwCVDkYgQPLPGYTTKGKEDvHcYsMQSk

...

sCQsELhRALeRLAASQ--S-RtH-EdLY\_IIPpNCdRNgnfHPKQCChPALdGqRGkCwCVD\_RKyGvKLPGGLEPKGEldChQLADsFRE

...

PCRRhMEASLQELKASPRMVpRAV----Y---LPNCdRkGFYkRkQCkPSrGRkRG\_IcWcVDkY-GmkLPGMEYVDGDfQCHtFDsSNvE

...

PCRRhLdsVLqqLqTEvY---RG-aQTLY---vPNCD\_HrGFYrKRQCrsSgQqRRGpCwCVD\_Rm-GKsLPGsPDgNGSSsCPTGSsG

Figure 3.iii. Amino acid sequences (from C to N termini) of human IGFBP-1 to IGFBP-6. Residues common to at least three of the binding proteins are shown as large upper case letters. The sequence of the IGFBP-3 cDNA probe described in this thesis is underlined. Taken from Shimasaki *et al* (1991).



untranslated region, an 873 bp coding region and approximately 1400 bp of 3' untranslated sequence. The mature binding protein comprises 264 amino acids and contains 18 cysteines, all of which are thought to be involved in forming disulphide bonds (Sommer *et al*, 1991), suggesting a potentially complex folding pattern. The IGFBP-3 has a 33% homology with IGFBP-1 but lacks an Arg-Gly-Asp (RGD) sequence suggesting no potential interaction with cell surface integrin receptors. The gene for IGFBP-3 has been located on chromosome 7 (Cubage *et al*, 1990).

### **Tissue expression and production**

A 2.4 kb IGFBP-3 mRNA species has been detected in many human and rat tissues, with particularly high levels of the gene observed in liver, ovary, spleen and prostate (Shimasaki *et al*, 1989; Naya *et al*, 1991). IGFBP-3 peptide has also been demonstrated *in vitro* in numerous cell types, including fetal, neonatal and adult human skin fibroblasts (Martin & Baxter, 1988, Camacho-Hubner *et al*, 1992), vascular endothelial cells (Bar *et al*, 1989), breast carcinoma (Adamo *et al*, 1992), and porcine and human ovarian granulosa cells (Mohan *et al*, 1989).

### **Regulation of IGFBP-3**

#### *In vivo* observations

The growth hormone (GH)-dependence of the 150 kDa complex has been known for a number of years, due to various chromatographic studies (Zapf *et al*, 1981), and a study demonstrating the reappearance of IGFBP-3 in hypophysectomised rats upon GH treatment (Moses *et al*, 1976). There is also a large body of evidence detailing a very close correlation between circulating IGFBP-3 levels and GH secretory status (Blum & Ranke 1991). For example, congenital and idiopathic hypopituitarism show reduced IGFBP-3 levels in line with their GH deficiency, whilst growth hormone excess in acromegalic patients is associated with markedly increased IGFBP-3, which decrease upon treatment (Hardouin *et al*, 1987).

As described earlier however, GH appears to be the primary mediator of IGF-I secretion,



particularly from the liver, and a series of more recent experiments has suggested that it may be the IGF-I peptide that directly mediates IGFBP-3 levels. Zapf *et al* (1989) showed IGF-I infusion into hypophysectomised rats (thus GH deficient) could itself restore IGFBP-3 to normal levels. IGF-I infusion into protein deficient (Clemmons *et al*, 1989) and diabetic rats, both GH resistant syndromes, can also significantly increase IGFBP-3 serum concentrations. Crucially, GH treatment was without effect in both of these models. Each of the above models do introduce additional variables other than GH deficiency however, such as removal of all anterior pituitary hormones, dietary imbalance, and potential disruption of insulin-mediated metabolic processes. To overcome these possible problems Camacho-Hubner *et al*, (1991) utilised a model consisting of four genotypically distinct groups of sibling transgenic mice that differed in respect to their expression of GH and IGF-I (Behringer *et al*, 1990). Low IGFBP-3 levels seen in their GH-deficient mice (caused by genetic ablation of somatotrophs) could be restored to near normal in similar animals expressing IGF-I having lost its GH dependence. Further to this, normal mice with genetically manipulated over-expression of IGF-I showed increased IGFBP-3, despite slightly reduced GH (probably due to an IGF-I negative feedback pathway acting on the pituitary). Thus, these experiments strongly implicated IGF-I as being the main regulator of IGFBP-3, at least in the rat.

Regulation of the 80 kDa acid-labile subunit however appears to be under direct control of GH itself. In the transgenic mouse model described earlier (Camacho-Hubner *et al*, 1991) those animals genetically GH deficient, but with normal expression of IGF-I, lacked the ternary 150 kDa complex despite having about two-thirds normal levels of IGF-I and IGFBP-3. More recent data has shown that synthesis of ALS by rat hepatocytes *in vitro* can be directly increased by the addition of GH (Scott and Baxter, 1991).

The *in vitro* fibroblast model.

Although a wide variety of cultured cell types have been shown to secrete IGFBP-3, human skin fibroblasts have been used most extensively in studies investigating the regulation of this binding protein. There are a number of reasons for this, including the ease at which they can be obtained, notably from the foreskins of newborn donors, their



successful maintenance *in vitro*, including serum-free environments, and their undifferentiated non-transformed phenotype.

The production and characterisation of IGFBPs released by human dermal fibroblasts has thus been well described over the last decade. Originally Adams *et al* (1984) reported the presence of an IGFBP complex of 150 kDa in fibroblast conditioned medium, which upon acidification became a lower molecular mass of between 50 - 60 kDa. This was consistent with the presence of the IGFBP-3 complex as seen in serum, with the IGFBP-3 peptide coupled to the 80 kDa acid labile subunit.

A more detailed study by Martin and Baxter (1988) revealed that in the absence of serum, neonatal skin fibroblasts secreted at least three IGFBPs. The largest and most abundant of these was indeed seen to cross-react with a specific antibody raised against the human plasma IGFBP-3, whilst not to a specific IGFBP-1 antiserum. Further, IGF binding studies showed the affinity of this peptide for IGF-I to be similar to that of plasma IGFBP-3. Its size estimation determined by affinity labelling and SDS Page also matched serum IGFBP-3, with the appearance of doublet between Mr 43,000 and 39,000 representing the two glycosylated forms of the binding protein.

Final confirmation of the presence of fibroblast derived IGFBP-3 was subsequently shown through recognition of IGFBP-3 mRNA using a full length human cDNA probe and Northern blotting (Camacho-Hubner *et al*, 1992). This study also clarified the identity of the other fibroblast IGFBP species. The 31,000 kDa IGFBP was shown to be IGFBP-5 whilst the smaller 24,000 kDa form corresponded to IGFBP-4.

Data from the initial experiments investigating the regulation of IGFBP-3 in these cells seemed to confirm many observations seen *in vivo*, with little or no change in IGFBP-3 secretion (measured by Western ligand blot) in response to GH. As outlined earlier there has been described *in vivo* a very close correlation between circulating IGF-I status and levels of IGFBP-3. Experiments analysing the conditioned medium from IGF-I and IGF-II treated fibroblasts indicated dose-dependent increases in IGFBP-3 levels (Clemmons *et al*, 1991), as was expected in view of the *in vivo* data. An IGFBP-3 radioimmunoassay confirmed these findings (Martin and Baxter, 1991) although increases of only fifty



percent were seen even after 72 hour incubations.

During these early studies it became apparent that despite the consistent increases in fibroblast IGFBP-3 in response to IGF, such an effect was not mediated via cell membrane receptors. Evidence against type-I IGF receptor involvement came from experiments using supraphysiological doses of insulin, capable of cross-reacting with this receptor, that were shown to be largely without effect (Clemmons *et al*, 1991). In addition, more recent studies using [QAYL]IGF-I, an IGF-I analogue with normal IGF receptor affinity but up to a six hundred fold reduced affinity for the IGFBPs, showed no effect of this peptide upon IGFBP-3 levels (Conover, 1991). Moreover, the type-I IGF receptor blocking antibody  $\alpha$ IR-3 caused no change in the native IGF-I effect. This apparently novel mechanism of IGFBP-3 regulation has now been reported to occur in a number of cell types, such as human and rat osteoblast-like cell cultures.

Data on the effect of IGF-I upon fibroblast IGFBP-3 gene expression has been more equivocal. One report has described IGF-I to inconsistently increase IGFBP-3 mRNA 20-60% but largely have no effect in human adult fibroblasts (Bale and Conover, 1992), thus supporting the mechanism described above for IGFBP-3 peptide regulation. However, another study has recorded a 2-fold increase in IGFBP-3 mRNA in response to IGF-I (Camacho-Hubner *et al*, 1992), although the cells were derived from a fetal source possibly explaining the discrepancy.

Certain other growth factors have also been shown to regulate IGFBP-3 secretion from fibroblast cells. Martin and Baxter (1991) demonstrated that epidermal growth factor (EGF) and transforming growth factor  $\beta$  (TGF $\beta$ ) induced increases in IGFBP-3 production from neonatal human skin fibroblasts during 72 hour incubations. Platelet derived growth factor (PDGF) similarly enhanced IGFBP-3 secretion in mouse 3T3 fibroblasts (Corps and Brown, 1991).

### **IGFBP proteases**

The assumption that the above regulators of IGFBP-3 operate by altering the molar amounts of secreted binding protein has been brought into question by the discovery of



proteolytic activity capable of degrading the IGFBPs (Hossenlopp *et al*, 1990). Such activity was initially described in the circulation of pregnant women and appeared to lead to the structural degradation of the IGFBPs, such that they exhibited a considerable reduction in affinity for  $^{125}\text{I}$ -IGF. Consequently, these patients gave low serum IGFBP-3 levels when assessed by Western ligand blotting, despite normal or even elevated values in immunological assays.

The presence of circulating proteolytic activity affecting IGFBP-3 has since been described in prostate cancer patients (Cohen *et al*, 1992), in children suffering GH deficiency (Cotterill *et al*, 1992) and in patients in a catabolic state (Davies *et al*, 1991). There is also evidence to suggest that this activity can be produced by a wide variety of transformed and non-transformed cells *in vitro* (Holly *et al*, 1993). Activity produced by rat prostate cells has been identified as a urokinase-type plasminogen activator (Koutsilieris *et al*, 1993), whilst in primates 72 and 96 kDa gelatinases have been implicated (Giudice *et al*, 1993).

At present, the functional significance of the IGFBP protease(s) is unknown and somewhat controversial, indeed at present there is little data suggesting specificity for the IGFBPs. However, studies in some *in vitro* systems have now shown that IGF bioactivity can be increased in the presence of IGFBP protease (Holly *et al*, 1993), perhaps indicating the potential importance of such a system for regulating IGFBPs.

### **Functions of IGFBP-3**

#### **Endocrine**

In normal human adults IGFBP-3 is the most abundant IGF binding protein in the circulation, with typical concentrations of 5 mg/l or 150 nmols/l (Baxter and Martin, 1986). Approximately 90% is found in the ternary complex of 140 kDa, resulting in restricted transport of the IGF peptide across the endothelial barrier (Binoux and Hossenlopp, 1988) and also a reduction in its renal clearance. Thus, the association of IGF with IGFBP-3 / ALS has been shown experimentally to greatly stabilise the IGF peptide, with its half-life in serum increasing over 50-fold, from about 10 minutes to 10 - 15 hours (Guler *et al*, 1989).



Moreover, as the formation of this complex is dependent upon the presence of the IGF peptide (on an equimolar basis), it follows that IGFBP-3 functions as a large circulating reservoir of IGF. Unlike most endocrine hormones the IGFs are synthesized and secreted somewhat constitutively from a wide variety of sources, rather than secreted acutely on demand from an inactive source stored in secretory granules. This has prompted the attractive hypothesis that the circulating reservoir of IGF held by the IGFBP-3 complex may act as a mobile endocrine 'gland', releasing the active peptide as required.

When one considers the difference between the resultant concentrations of the IGFs and those of insulin (some 100 : 1, in respective molarities) we must assume that most of the IGFs within the IGFBP-3 complex have been inactivated, otherwise severe hypoglycaemia would surely occur. Increases in IGF activity however, are observed in cases of high IGFBP-3 levels, such as acromegaly, due to the parallel increase in IGF peptide. Indeed, an *in vivo* animal model of wound repair found that treatment with IGF and IGFBP-3, when compared with IGF-I given alone, resulted in enhanced tissue formation (Sommer *et al*, 1991). This suggests that although IGFBP-3 seems to act as an IGF inhibitor, the rate of IGF release from the complex to the tissues remains constant allowing alterations in steady state IGF levels, dependent upon IGFBP levels, to be reflected in concomitant changes in bioactivity. The specific mechanisms which regulate the release of IGF from the IGFBP-3 are at present largely unknown, although heparin and certain glycosaminoglycans have been shown to dissociate the binary IGF/IGFBP-3 complex from the acid labile subunit (Baxter, 1990).

#### Autocrine / paracrine

In addition to extravascular IGFBP-3 derived from an hepatic origin and transported across the endothelium, a large amount of IGFBP-3 found amongst the tissues is likely to be from a more local source, either autocrine or paracrine. Moreover, whilst certain interpretations on the function of circulating IGFBP-3 can be made from the large number of *in vitro* studies carried out on this binding protein, the data obtained is perhaps more pertinent towards our understanding of this more local source of IGFBP-3.

Most of the IGFBP-3 data from cell culture *in vitro* studies have been in agreement with



its supposed function in the circulation, i.e. an inhibitor of IGF activity. Initial studies using impure preparations of serum IGFBPs (mostly IGFBP-3 therefore) found inhibition of IGF actions ranging from stimulation of glucose transport in adipocytes (Chochinov *et al*, 1977), sulphate incorporation by chondrocytes (Zapf *et al*, 1979), and thymidine incorporation in fibroblasts (Drop *et al*, 1979). A later study by DeMellow and Baxter (1988) also observed IGF-I inhibition when the peptide was co-incubated with pure IGFBP-3, presumably by preventing it from associating with the IGF cell membrane receptors.

Such a simplistic understanding of the function of IGFBP-3 was however brought into question from other data presented in this same study. A *pre-incubation* of the fibroblast cells with the pure IGFBP-3 preparation prior to the addition of IGF-I, was found to result in up to a 50% *increase* in subsequent IGF-I activity. Others have now reported similar IGF-I responses, following these particular incubation conditions, with both glycosylated and non-glycosylated IGFBP-3 (Conover, 1991). Although the precise mechanism involved is unknown, IGFBP-3 sensitisation of cells by blocking endogenous IGFs from down-regulating membrane receptors has been suggested, as has cell bound IGFBP-3 directly modulating the presentation of the IGF molecule to its receptor.

### **1.8. Insulin-like growth factor binding-protein 1**

#### **Characterisation**

IGFBP-1 was first detected in and purified from amniotic fluid (Chochinov *et al*, 1977; Drop *et al*, 1979). It was also the first IGFBP to be cloned (Lee *et al*, 1988) and its gene characterised (Brinkman *et al*, 1988). In humans this binding protein consists of 259 amino acids with a calculated molecular weight of approximately 28.1 kDa, is highly negatively charged and rich in cysteine residues.

The IGFBP-1 gene spanning 5.2 kb is found on chromosome 7, located just 20 kb from the IGFBP-3 gene. This is reflected in their respective protein coding regions, with homologous exons 1,3 and 4, and a dissimilar exon 2. The N-terminal portion of IGFBP-1 is thought to be responsible for IGF binding whereas the hydrophylic C-terminal region



contains an RGD sequence (Arg-Gly-Asp tripeptide) which represents a potential cell attachment site similar to that found in the integrin family of proteins (Brewer *et al*, 1988).

Although having no glycosylation sites, IGFBP-1 is at present thought to be unique amongst IGFBPs in that it can exist in a number of differentially phosphorylated forms (Jones *et al*, 1991). Human hepatoma (HepG2) cells and human decidual cells secrete at least four different isoforms, with binding affinities for IGF-I between 3 and 7-fold greater than the non-phosphorylated form. The presence of a specific phosphatase in amniotic fluid and fetal serum has been suggested after the appearance of non-phosphorylated and phosphorylated forms, and may be an important regulator of IGFBP-1 function (see below).

### **Tissue expression and production**

The main site of IGFBP-1 production is the liver, and although the peptide is widely distributed throughout many other tissues, other sources of IGFBP-1 mRNA are restricted. It has been identified in human amniotic fluid as a 35 - 40 kDa binding protein (named *AFBP*), in tissue extracts of fetal and maternal placenta (*placental protein 12*, Koistinen *et al*, 1986) and in the conditioned medium of human HepG2 hepatoma cells (*BP-25*, Lee *et al*, 1988).

Due to its early discovery and description IGFBP-1 has historically been assigned a multitude of pseudonyms. IGFBP-1 in human pregnancy endometrium has been variously described as pregnancy-associated endometrial  $\alpha^1$  globulin ( $\alpha^1$ -*PEG*), chorionic  $\alpha^1$ -microglobulin (*CAG-1*), placental-specific  $\alpha^1$ -microglobulin (*PAMG-1*) and endometrial protein 14 (*EP14*). Analysis demonstrating the homology between these independently isolated proteins together with the introduction of a standardised nomenclature (Ballard *et al*, 1989) brought all these terminologies under the single designation of IGFBP-1.

### **Regulation of IGFBP-1**

#### *In vivo*

In normal human adults IGFBP-1 has the third highest serum concentration amongst the



IGF binding proteins in normal human adults, although its typical levels (5 nmol/l) are approximately 50-fold less than IGFBP-3. Unlike IGFBP-3 however, this binding protein is not regulated by GH and undergoes a marked diurnal variation with its highest levels detected in the morning (Cotterill *et al*, 1988) reaching a typical peak of 100 nmol/l. This acute variation has since been shown to be inversely related to the secretory pattern of insulin (Holly *et al*, 1988). Insulin dependence of IGFBP-1 has now been observed in a number of metabolic disorders characterised by abnormal insulin status. Increased serum IGFBP-1 has been described in GH deficiency and anorexia, conditions of low insulin, while raised IGFBP-1 has been recorded in obesity, polycystic ovarian syndrome, acromegaly and diabetes.

### *In vitro*

Much of the *in vitro* data on the regulation of IGFBP-1 has come from studies involving the HepG2 human hepatoma cell line. In agreement with the *in vivo* data, the prodigious secretion of IGFBP-1 by these cells is reduced when incubated with insulin (Cotterill *et al*, 1989) and IGF-I, both apparently operating through their respective receptors. Conversely, increased hepatic IGFBP-1 secretion can be found in conditions of low glucose, such as the specific inhibition of hexose uptake by cytochalasin B. Factors which stimulate intracellular cyclic nucleotide accumulation also have this effect.

## **Functions of IGFBP-1**

### Endocrine

The regulatory patterns of IGFBP-1 seen *in vivo* suggest that this binding protein is important in controlling the acute actions of the IGFs. Moreover, its response to glucose administration and experimentally induced hypoglycaemia are consistent with a role in glucose counter-regulation, principally through its inhibitory effect on IGF bioactivity, as seen in the *in vitro* studies. As approximately 98% of circulating IGF is held within the 140 kDa IGFBP-3 complex, it has been hypothesized that the effect of acute increases in IGFBP-1 is to block the 'free' IGF fraction (some 2 nmol/l) thus preventing its insulin-like activity. Other studies have ascribed the role of an IGF transport molecule to IGFBP-



1, carrying the IGF from the circulation to the tissues. Primarily this has arisen from data describing the ability of IGFBP-1 to traverse the endothelial barrier (Bar *et al*, 1990), thereby explaining the widespread nature of the peptide when compared to its sites of mRNA expression.

#### Autocrine / Paracrine

The precise function of non-hepatic IGFBP-1, such as that from endometrial tissue, is controversial. Most *in vitro* studies have shown that exogenous IGFBP-1 inhibits both the metabolic and mitogenic actions of the IGFs, an effect thought to be due to its ability to compete with the cell membrane receptors (Ritvos *et al*, 1988).

Conversely however, others have described IGFBP-1 mediated enhancement of IGF bioactivity (Elgin *et al*, 1987), and unlike that seen in response to IGFBP-3 this can occur when both peptides are co-incubated. This effect has been associated with the adherence of the binding protein to target cell surfaces (Busby *et al*, 1988), perhaps working through its RGD amino acid sequence interacting with the integrin receptor family. Interesting data has also suggested that the degree of phosphorylation of IGFBP-1 may play a central role in affecting its function as an IGF regulator at the tissue level (Clemmons *et al*, 1991). The HepG2 cell line, for example, secretes at least four differentially phosphorylated isoforms, each consistently inhibiting the mitogenic effect of IGF-I. Dephosphorylated IGFBP-1 however potentiates IGF-I action, possibly through a reduction in affinity for the growth factor, indicating that a potential mechanism of regulating its cellular activity may indeed be via dephosphorylation, by a specific phosphatase for example.

As more data is obtained on this dichotomous function of IGFBP-1 the role of this binding protein in regulating IGF bioactivity, at least at the tissue level, seems certain to be modified from the simple inhibitor as it was first ascribed.

### **1.9. Insulin-like growth factor binding-protein 2**

#### **Characterisation**

One of the earliest IGFBPs to be discovered was IGFBP-2, first identified as a binding



protein for Multiplication Stimulating Activity (MSA), or rat IGF-II, in culture media conditioned by the rat liver cell line BRL-3A (Romanus *et al*, 1986). The precise primary structure was subsequently determined from cDNA clones isolated from the adult rat liver (Margot *et al*, 1989, Binkert *et al*, 1989). The mature peptide is comprised of 270 amino acids, with no glycosylation sites, resulting in a molecular weight of 29.5 kDa. Cysteine rich domains are present however, and like IGFBP-1 it contains the RGD sequence of amino acids, suggesting possible association with the integrin receptor family. The primary structure of IGFBP-2 is conserved amongst species, with human and rat species sharing 90% sequence homology. Its gene has been located on chromosome 2 (Shimasaki *et al*, 1990) and codes for a single mRNA transcript of 1.6 kb.

### **Tissue expression and production**

As perhaps expected from its initial discovery in rat hepatocytes *in vitro*, the major source of circulating IGFBP-2 is from the liver. Typically, this binding protein is the second most abundant IGFBP in the circulation, with levels of 20 nmol/l, and remains fairly constant throughout the day (Clemmons *et al*, 1991). The production of IGFBP-2, both from a developmental perspective and in its restriction to certain cell types other than those of hepatic origin, seems to be linked to the presence of IGF-II. For example, this binding protein is especially predominant in early fetal life and subsequently declines into adult life, thus following the pattern of IGF-II. It is also associated with a number of IGF-II secreting tumours such as rhabdomyosarcomas (Roghani *et al*, 1989), human breast carcinoma (DeLeon *et al*, 1989), and also in non-islet cell tumour hypoglycaemia (Zapf *et al*, 1990). IGFBP-2 is the predominant IGFBP found in human cerebro-spinal fluid (CSF), and has also been observed in medium conditioned by cultured fetal neuronal and astroglial cells (Lamson *et al*, 1989).

### **Regulation**

Due to its close correlation with circulating IGF-II levels, it has been suggested that this growth factor may be the primary regulator of this binding protein. Despite certain similarities with IGFBP-1, its pattern of regulation appears to be distinct. In the human



subject acute stimulation of insulin has little or no effect upon circulating levels of IGFBP-2, although in severe insulin deficiency (such as fasting) levels can be increased. Moreover, in rats (where IGF-II is perhaps of greater importance) raised hepatic IGFBP-2 is found in diabetic animals, reducing upon treatment with insulin (Boni-Schnetzler *et al*, 1990).

### **Functions of IGFBP-2**

The precise function of IGFBP-2 is unclear. It has also been suggested that IGFBP-2 seems to be a 'reserve' binding protein in the circulation, acting to provide replacement IGF binding sites in conditions where the IGFBP-3 ternary complex is reduced. Thus, its circulating levels are high in hypopituitarism and fasting patients, reflecting the distinct mode of regulation seen with this particular IGFBP. Its close association and preferential affinity for IGF-II, some ten fold greater than that for IGF-I in the rat (Forbes *et al*, 1988), also suggests that it may have a specific role in mediating the physiology of this IGF species. However, as the function of IGF-II itself is still largely unknown we can only speculate as to its importance. As with both IGFBP-1 and IGFBP-3, exogenous IGFBP-2 can inhibit IGF activity *in vitro* (Han *et al*, 1988), although no data has yet been presented on its potential capacity to enhance such activity.

### **1.10. Insulin-like growth factor binding-protein 4**

#### **Characterisation**

The low molecular weight IGFBP-4 was first identified in human serum (Hardouin *et al*, 1987) and has subsequently been isolated and sequenced from human and rat sources (Shimasaki *et al*, 1990; LaTour *et al*, 1990). The human cDNA is 1899 base pairs in size and encodes a 237 amino acid mature protein, with a predicted molecular weight of 25,970 daltons. Its gene is found on chromosome 17 and results in the expression of a single 2.2 kb mRNA transcript. The 18 cysteine residues common to IGFBP-1, -2, and -3 are retained, but there also exists an additional two cysteines at the mid portion of the molecule. The molecule has the potential to exist in a glycosylated form, with a single N-



linked glycosylation site, but does not contain the RGD amino acid sequence seen in both IGFBP-1 and -2. Historically, IGFBP-4 has been named both Binding Protein 24 (BP24) and Bone-Cell Derived Binding Protein.

### **Tissue expression and production**

IGFBP-4 was originally identified in the conditioned media from human osteosarcoma and prostatic tumour cells (Mohan *et al*, 1989; Perkel *et al*, 1990). It has since been described from a number of other cell types *in vitro*, including human skin fibroblasts (Conover *et al*, 1991) and human breast cancer cells (De Leon *et al*, 1989). In the rat neuroblastoma cell line B104 a 28 kDa glycosylated IGFBP-4 variant has been observed in addition to the more usual non-glycosylated 24 kDa peptide (Ceda *et al*, 1991). IGFBP-4 mRNA expression is most abundant in the liver, although has been recorded in many other tissues such as adrenal, testis, spleen, heart, lung, kidney and stomach (Shimasaki *et al*, 1990).

### **Regulation of IGFBP-4**

Parathyroid hormone (PTH), which inhibits the formation of bone both *in vivo* and *in vitro*, can regulate IGFBP-4 by enhancing mRNA and subsequent protein levels in cultures of normal human osteoblast-like cells (La Tour *et al*, 1990). In contrast, the IGFs can inhibit the appearance of IGFBP-4 in the conditioned media of human fibroblast cultures (Neely and Rosenfeld, 1992) although in a similar fashion to the IGF induced enhancement of IGFBP-3 in these cells, this effect is not mediated via the type I or type II IGF receptors, and does not involve modulation of IGFBP-4 gene expression (Camacho-Hubner *et al*, 1992).

### **Functions of IGFBP-4**

Little is known of the function of IGFBP-4. Its enhancement by PTH which can be catabolic in bone may be consistent with the generally held view of IGFBPs, that of acting as inhibitors of IGF (anabolic) activity. However, certain *in vivo* studies have also indicated that PTH may actually increase osteoblast growth and have an overall anabolic action in new bone formation, possibly through the mediation of secondary factors such



as the IGFs (Tam *et al*, 1982). To clarify the role of IGFBP-4 in this system more studies are required to determine if IGFBP-4 can enhance IGF actions in a similar fashion to IGFBP-1 and -3, as described earlier.

Although the function of glycosylated residues on IGFBP-3 has yet to be determined, their presence on certain IGFBP-4 molecules has prompted some to suggest that these may be serum IGFBPs, with non-glycosylated subpopulations serving different functions at the tissue level.

### **1.11. Insulin-like growth factor binding-protein 5**

#### **Characterisation**

IGFBP-5 consists of 252 amino acids, with the 18 cysteine residues characteristic of other IGFBPs evident, giving a predicted molecular weight of 28.5 kDa. The RGD amino acid sequence is lacking although the protein may exist in an O-glycosylated form. The gene for IGFBP-5 is located on chromosome 5, and gives rise to a single mRNA species of 6.0 kb (Shimasaki *et al*, 1991).

#### **Tissue expression and production**

IGFBP-5 mRNA is most abundant in the kidney, but is also found in the intestine, adrenal, heart, lung, brain, spleen. It is almost absent from the liver, resulting in undetectable levels in serum. A number of cells *in vitro* also produce this IGFBP, including a human glioblastoma tumour cell line designated T98G (from which the peptide was originally purified and sequenced), osteoblast-like cells (Andress and Birnbaum, 1991) and also human fetal fibroblasts (Camacho-Hubner *et al*, 1992). It is also the only IGFBP to be produced by the untransformed rat thyroid cell line FRTL-5 (Backeljauw *et al*, 1993).

#### **Regulation of IGFBP-5**

Levels of IGFBP-5 increase by six to eight-fold in media conditioned by human fibroblasts following 24 hour incubations with maximal doses of IGF-I or IGF-II



(Camacho-Hubner *et al*, 1992). This effect was not however mediated through either IGF or insulin cell membrane receptors, but was directly dependent upon the IGF peptide binding to the IGFBP. Such post-transcriptional regulation has also been observed for IGFBP-3, and may involve the IGF displacing IGFBP-5 from the cell membrane or protection from proteolytic activity. IGF-I increases IGFBP-5 mRNA and peptide in the non-transformed rat thyroid cell line FRTL-5, and insulin, via its own receptor, also seems to increase IGFBP-5 in these cells.

### **Functions of IGFBP-5**

Due to its absence from serum the function of IGFBP-5 is presumed to be limited to affecting the IGFs at the tissue level. In fibroblasts it is preferentially localised in the extracellular matrix (Jones *et al*, 1993), apparently binding to specific components, despite lacking the RGD amino acid sequence recognised by the integrin receptor family. The precise function of these additional sites for cell surface localisation of the IGF peptides is unknown at present. In the osteoblast-like cells however IGFBP-5 has been demonstrated to potentiate the mitogenic activity of IGF-I.

## **1.12. Insulin-like growth factor binding-protein 6**

### **Characterisation**

The last IGFBP to be discovered, termed IGFBP-6, was originally isolated from human cerebrospinal fluid (CSF) where it constitutes the predominant binding protein (Roghani *et al*, 1989). Subsequently, it was cloned and characterised by Shimasaki *et al* (1991), using porcine follicular fluid and adult rat serum. Human IGFBP-6 consists of 216 amino acids, with a predicted molecular weight of 22.8 kDa, and lacks both the short RGD sequence recognised by the integrin cell surface receptors, and 2 of the 18 cysteines characteristic of all other IGFBPs (**figure 1.iii.**). IGFBP-6 typically exists in a glycosylated form similar to IGFBP-3 and -4, although unlike the latter it is O-glycosylated rather than N-glycosylated (Bach *et al*, 1993). Rat IGFBP-6 is similar in structure (201 amino acids) and size (21.5 kDa) but lacks 4 cysteines. The gene for



IGFBP-6 is located on chromosome 12, and encodes for a single 1.3 kb mRNA transcript.

### **Tissue expression and production**

IGFBP-6 mRNA has been found in a wide range of tissues, including the liver, resulting in typical serum peptide levels in normal human adults of approximately 10 nmol/l (Baxter and Saunders, 1992), comparable to those of IGFBP-2 and higher than baseline IGFBP-1. In addition, it has been purified from ovarian follicular fluid, CSF, and from the conditioned media from transformed and non-transformed human lung fibroblasts.

### **Regulation of IGFBP-6**

Little attention has been given to the regulation of IGFBP-6 *in vivo*, perhaps due in part to its relatively recent discovery and its identity on Western ligand blots obscured by similar sized IGFBPs. Data from a specific radioimmunoassay (Baxter and Saunders, 1992) has revealed levels of IGFBP-6 are generally higher in men than women, and are significantly reduced in pregnancy and in patients with active acromegaly.

### **Functions of IGFBP-6**

IGFBP-6 has up to a 30-fold lower affinity for IGF-I compared to IGF-II (Kiefer *et al*, 1993). Data from *in vitro* studies, using exogenous recombinant non-glycosylated IGFBP-6, has shown that this results in the binding protein being much more effective at inhibiting the mitogenic activity of the latter growth factor. The functional significance of the unique O-glycosylation pattern is unknown.

## **SUMMARY**

The large number of studies described above demonstrate that the IGFBP family provides us with a complex network of potential IGF regulators, in addition to the more usual pathways of altered IGF gene expression and IGF cell membrane receptors. However, whilst our knowledge of IGFBP structure is now fairly well defined, with gene structure, sites of expression and amino acid sequences deduced, there is certainly more scope for

studies investigating regulation of IGFBP secretion and their biological function, particularly at the tissue level. The recent discovery of IGFBP protease activity may also implicate certain novel regulators hitherto overlooked. It is these aspects of IGFBP physiology that we have tried to address in the following thesis, principally through the development of a human fibroblast *in vitro* model and investigation of potential IGFBP and/or IGFBP protease regulators. In this model we have therefore studied certain members of a large group of peptides, the cytokine family, that have well recognised effects upon both tissue growth and protease activity. A brief introduction to these peptides is now given.



# **CHAPTER 1.**

## **SECTION C. THE CYTOKINE FAMILY**

### **Overview**

As I have described in the previous section, an increasing number of hormones and growth factors have been shown to affect the production of IGFBPs both *in vitro* and *in vivo*. However, little data exists on their possible regulation by a major group of regulatory peptides known as the cytokines. This is rather surprising because contained within this group are a number of factors which have a dramatic impact upon the growth of a wide range of cell and tissue types. Moreover, many of their actions can be considered to be indirect, often being ascribed to the stimulation of secondary factors in the given cell type, and frequently involve the alteration in the secretion of globular proteins, such as albumin and various extracellular matrix components. In addition, they also often modulate metalloproteinase production and their associated inhibitors in many *in vitro* cell culture models. These characteristics thus make them good candidates to be IGFBP regulators.

There is some recent evidence that at least one member of this family, which includes the inflammatory lymphokines, the transforming growth factors and the ever expanding interleukin group, is capable of affecting IGFBP production *in vitro*. Specifically, treatment of normal human skin fibroblasts with transforming growth factor  $\beta$  (TGF $\beta$ ) was seen to lead to a significant increase in IGFBP-3 secretion from such cells (Martin and Baxter, 1991). In the following study this response has been further characterised and the effects of tumour necrosis factor  $\alpha$  (TNF $\alpha$ ), interleukins 1 $\alpha$  and 1 $\beta$  (IL-1 $\alpha$  and IL-1 $\beta$ ) and interleukin-6 (IL-6) investigated. An introduction to these factors is given below.

### **1.13. Transforming growth factor $\beta$ (TGF $\beta$ )**

#### **Background**

Transforming growth factor (TGF) was a term originally applied to describe an activity present in media conditioned by a murine sarcoma virus-transformed cell line (DeLarco and Todaro, 1976). This activity was characterised by its ability to induce the growth of



normally anchorage-dependent cells in soft agar, a hallmark of *in vitro* transformation. Although it is now apparent that this effect is but one of their many functions, it has been accepted as the operational definition of the family of TGF-like peptides.

Two classes of TGF exist, designated TGF $\alpha$  and TGF $\beta$ , although other structurally related polypeptides include the inhibins and the Mullerian inhibitory substance. Although given similar names TGF $\alpha$ , an epidermal growth factor-like peptide, and TGF $\beta$  are however distinct molecules in structure and function. In the context of the present study we are concerned only with TGF $\beta$ , a brief introduction of which is now given.

### **Primary structure**

TGF $\beta$  is a dimeric polypeptide comprised of identical 112 amino acid subunits, linked by a series of disulphide bonds. A number of isoforms, all retaining this characteristic feature, have now been identified and are designated TGF $\beta$ 1, 2, 1.2, 3 and 4. The first of these was purified from human platelets (Assoian *et al*, 1983) and human cDNA clones subsequently isolated (Derynck *et al*, 1985). It exists as a 25 kDa dimer comprising the two identical 12.5 kDa subunits, each derived from cleaved 390 precursor peptides. TGF $\beta$ 2 is a similar dimeric peptide with subunits showing a 72 % sequence homology with TGF $\beta$ 1. TGF $\beta$ 1.2 is a heterodimer containing one TGF $\beta$ 1 and one TGF $\beta$ 2 chain. At present there is little evidence to suggest functional divergence among the various isoforms.

### **Tissue expression and production**

TGF $\beta$  mRNA and peptide has been detected in a wide range of normal and transformed tissues and cell types both *in vivo* and *in vitro*. In adult mammals TGF $\beta$  is particularly high in platelets and bone chondrocytes (Robey *et al*, 1987), whilst circulating levels may typically reach 5  $\mu$ g/l (Shirai *et al*, 1992). TGF $\beta$  can also be found in macrophages and peripheral blood monocytes, and its expression seems to correlate with tissue developmental stage, with highest levels observed during periods of morphogenesis (Akhurst *et al*, 1990).



## **Biological functions**

Like many other cytokines TGF $\beta$  is a multifunctional regulator of cellular activity. Responses are mediated via three structurally distinct cell membrane high affinity receptors, designated type I, II and III, with molecular weights of 65, 85-110 and 560 kDA respectively. All three may be expressed simultaneously, usually in a constitutive manner, in epithelial, mesenchymal and haematopoietic cells of both normal and tumoural origin. Although not fully elucidated, signal transduction appears to involve one or more GTP-binding proteins (Kataoka *et al*, 1993).

Generally, TGF $\beta$  is a potent, but reversible, inhibitor of normal cell growth *in vitro* stimulated by serum or growth factors, including the IGFs (Zugmaier *et al*, 1989). The effect of TGF $\beta$  on cell proliferation is however often bifunctional and can be markedly influenced by other growth factors. In normal fibroblasts for example, inhibition may be seen with picomolar doses in subconfluent cultures, although conversely may actually stimulate growth in quiescent monolayers. A similar bimodal effect is also seen in bone osteoblast cultures.

Other functions include a pronounced effect on extracellular matrix production (for review see: Noble *et al*, 1992). It causes an increase in collagen, fibronectin and proteoglycan expression, an increase in integrin expression, a decrease in proteinase secretion, including collagenase and transin, and also increases proteinase inhibitors such as plasminogen activator inhibitor (PAI-1) and tissue inhibitor of metalloproteinases (TIMP). It has been suggested that such a co-ordinated effect is designed to aid the process of wound healing, a hypothesis encouraged by the high TGF $\beta$  expression in platelets, and its ability to enhance epidermal keratinisation and chemotaxis. TGF $\beta$  also has marked immunoregulatory functions, generally acting as a down-regulatory stimulus in immune responses.

### **1.14. TUMOUR NECROSIS FACTOR $\alpha$**

#### **Background**

For over 100 years there have been reports in the cancer literature of tumour necrosis and



regression after spontaneous or experimentally induced bacterial infection. Indeed, 'Coley's mixed toxins', a filtrate of bacterial cultures, were described by the American Medical Association in 1934 as the only known systemic therapy for cancer (Old, 1986). Further research identified the factor responsible for eliciting such tumour necrosis as the lipopolysaccharide constituent of bacterial cell walls, named endotoxin. Carswell *et al* (1975) subsequently described the cytokine tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) as the serum factor produced by the host in response to bacterial endotoxin.

### **Structure**

Isolated TNF $\alpha$  cDNA is a 233 amino acid chain, containing a 76 residue pre-sequence which is lost upon maturation and thought to be important in macrophage secretory processes. TNF $\alpha$  peptide is first produced as a propeptide, which is cleaved to yield the active peptide. The mature protein therefore consists of 157 amino acids with no glycosylation sites, resulting in a molecular weight of 17,356 daltons. Cysteine residues are present however, forming a number of interchain disulphide bonds. Its gene is found on human chromosome 6 mapped in the major histocompatibility complex (MHC) encoding region.

### **Tissue expression and production**

TNF $\alpha$  was first purified from the culture supernatant of the human pro-myelocytic leukaemic cell line HL-60, although it is now known to be produced by a wide variety of immune cells including macrophages, T cells, thymocytes, B cells and natural killer (NK) cells. When suitably induced monocytes can secrete large amounts of TNF $\alpha$ , up to 1% of their total secretory product for 8 hours or more (Gifford and Flick, 1987).

TNF $\alpha$  can be induced by a number of agents other than endotoxin, including a number of different viruses and the tumour-promoting agent PMA. In addition, certain other cytokines can stimulate or augment the secretion of TNF $\alpha$ .

### **Biological actions of TNF $\alpha$**

To exert their cell regulatory or cytotoxic effects, TNF $\alpha$  must first interact with specific



cell surface membrane receptors. Two classes of high-affinity receptors, TNF-R-75 and TNF-R-55, have now been reported to exist on a variety of normal and transformed cells (Kull *et al*, 1985). The nature and second messenger of the signal transduced by these receptors is not fully understood and is the subject of intense interest. Recent reports have however suggested the involvement of certain protein kinase cascades, phosphorylating specific receptor proteins (Kalthoff *et al*, 1993). Depending on the particular cell type, binding of TNF $\alpha$  to the membrane receptors can result in a multiplicity of effects. For convenience only, we can assign these into three groups, although they are most certainly inter-related.

A major function of TNF $\alpha$ , and the one which lead to its initial discovery, is to mediate immune directed cytotoxicity. It causes lysis of certain tumours *in vivo*, such as Meth-A sarcomas (Gray *et al*, 1984), and also a number of transformed cell lines, including mouse L-929 fibroblasts, *in vitro* (Matthews, 1978). TNF $\alpha$  cytotoxicity can be independent of protein synthesis and can be blocked by protease inhibitors, suggesting that one or more proteases induced by TNF $\alpha$  may either directly cause cell damage or may activate other lytic enzymes (Ruggiero *et al*, 1987). The presence of metabolic inhibitors or interferon  $\alpha$  and  $\sigma$  seem to enhance the cytotoxic effect of TNF $\alpha$  in a wide range of cell types. It is also notable that many cell types that fully express TNF $\alpha$  receptors are not killed by the cytokine, although receptor number on susceptible cells is proportional to the amount of cell lysis.

Contrasting with the effects described above, TNF $\alpha$  can also act as a growth factor *in vitro*, stimulating mitogenesis in normal diploid fibroblasts and certain tumour cells (Vilcek *et al*, 1986; Dealtry *et al*, 1987). Although TNF $\alpha$  may function as a direct mitogen, there is evidence in a normal human skin fibroblast model that it may act synergistically with other serum growth factors, particularly insulin (Vilcek *et al*, 1986). TNF $\alpha$  stimulation of cell growth has been demonstrated at concentrations comparable to those shown to produce cytotoxicity in many transformed cell lines.



In addition to its effect on cell proliferation TNF $\alpha$  is also thought to have profound effects on the secretion of a wide range of proteins involved in inflammation and immunity. It can for example induce interleukin-1 and -6, interleukin 2 receptors, prostaglandin E<sub>2</sub>, and a number of metalloproteinases. It can also inhibit the production of albumin, transferrin, lipoprotein lipase and collagen, implicating it in the mediation of connective tissue imbalance.

### **1.15. INTERLEUKIN-1 and 6**

The term interleukin was originally coined to describe molecules that sent signals between leucocytes. However, further understanding of these cytokines has proved this definition to be rather limiting, as they can often be produced by and have regulatory effects upon a wide variety of other cell types. Consequently, since 1986 all newly discovered cytokines have also been ascribed an interleukin number, provided amino acid sequence analysis has proven them to be novel. At present, this large and expanding family of cytokines now spans interleukin 1 to 13, and includes a whole variety of isoforms and subtypes. Obviously, in the context of this thesis it is impractical to examine the effects of each interleukin on the IGF / IGFBP axis, so experiments were restricted to the study of those that have been shown to have close functional similarity with TNF $\alpha$ , namely interleukin-1 and -6.

#### **1.15.i. Interleukin 1**

##### **Structure**

There are two different IL-1 genes coding for two proteins, named IL-1 $\alpha$  and IL-1 $\beta$ , which bind to the same cell surface receptor and consequently have identical biological actions (Lomedico *et al*, 1987). Both arise from large 35 kDa precursor molecules comprising 271 (IL-1 $\alpha$ ) and 269 (IL-1 $\beta$ ) amino acids, and are processed into 17.5 kDa mature peptides of 159 and 153 amino acids, respectively. Sequence homology between the two species is however restricted to 27%, although as this is contained in the carboxy-terminus it seems sufficient to confer identical receptor recognition sites.



## **Tissue expression and production**

In addition to the traditional haematopoietic sources of IL-1, such as that from monocytes, macrophages, T and B lymphocytes and NK cells, it is now accepted that most cell types can produce certain amounts of both IL-1 species, although often only when stimulated by mitogens (Oppenheim *et al*, 1986). In a similar manner to TNF $\alpha$ , IL-1 $\alpha$  and IL-1 $\beta$  can be induced by antigen, toxins, injury, inflammatory processes, and other cytokines including TNF $\alpha$  and the interferons.

## **Biological actions**

Membrane receptors for IL-1 have been found on a wide range of cell types, with binding to a 80 kDa species mediating the biological actions of the cytokine. Signal transduction is similar to that of the TNF $\alpha$  receptors and appears to involve the activation of serine / threonine kinase activity independent of protein kinase A or C. Such similarity may help explain its close functional relationship with TNF $\alpha$ .

The biological effects of IL-1 are largely dependent upon the particular cell type in question. It plays a major role in positively regulating the activity of many haematopoietic cells involved in mediating immunity, including T and B lymphocytes, and is thus responsible for many of the systemic manifestations of infection (Dinarello and Mier, 1986). However, it also has wide ranging actions in other cell types perhaps more closely linked with the IGFs. In bone, for example, IL-1 $\beta$  is an osteoclast-activating factor and leads to the inhibition of new proteoglycan synthesis in cartilage. In a similar manner to TNF $\alpha$ , IL-1 also has the conflicting property of being mitogenic for some cells and cytostatic or cytotoxic for others. In fibroblasts, IL-1 is an extremely potent mitogen (Schmidt *et al*, 1982) and also increases the synthesis of procollagen, collagenase, tissue inhibitor of metalloproteinases (TIMP) and prostaglandin E<sub>2</sub>, perhaps suggesting an involvement in tissue repair. It can be both mitogenic and cytotoxic in tumour cells.

### **1.15.ii. Interleukin 6**

#### **Structure**

IL-6 has previously been known as B cell stimulatory factor (BSF-2), interferon- $\beta$ 2, 26

kDa protein, and hepatocyte stimulating factor (HSF). The cDNAs code for a protein consisting of 212 amino acids (22 - 29 kDa) that has two potential glycosylation sites and four cysteine residues (Hirano *et al*, 1989).

### **Tissue expression and production**

Although the major source of circulating IL-6 is of haematopoietic origin, principally from the mononuclear T-cells, it now appears that it may also be produced by a wide range of other cell types, such as fibroblasts (Van-Damme *et al*, 1987) and epithelial keratinocytes. The IL-6 gene can be induced by inflammation-associated cytokines, such as TNF $\alpha$  and IL-1, bacterial products, viral infections, and activation of either cAMP, calcium ion or diacylglycerol signal transduction pathways.

### **Biological actions**

IL-6 activity is mediated via its own specific cell receptors. These consist of two membrane proteins, a ligand-binding chain (designated IL-6R) and a non-ligand binding signal transducer (designated gp130) which associate once in the presence of IL-6 and activate protein kinases (Kishimoto, 1992).

IL-6 acts on a wide variety of cells regulating the immune response, acute phase reaction and haematopoiesis. That made by activated T cells for example has the capacity to act as B-cell differentiation factor by stimulating human B cells to secrete antibody. At present, little data exists on the relationship between IL-6 and the IGFs.



## **CHAPTER 1.**

### **SECTION D. AIMS OF THESIS**

The principle objectives of this thesis were to investigate three aspects of IGFBP physiology at the cellular level.

1. To establish the possible mechanisms involved in the regulation of IGFBP-3 secretion, *in vitro*.

In addition to the classical pathway of modulating IGFBPs, that of altered gene expression, it is becoming increasingly apparent that a number of post-translational mechanisms may also exist. In the normal human fibroblast cell culture model, such mechanisms may be involved in IGF-I regulation of IGFBP-3, as this appears to be a non-receptor mediated event. Using a normal neonatal human foreskin fibroblast cell line, designated Hs68, the effect of IGF-I on IGFBP-3 has been analysed by Western ligand blotting, radioimmunoassay and Northern blotting, and assessment made of the role that the recently discovered IGFBP proteases may have in mediating this effect.

2. To investigate the effect of cytokines on IGFBP production *in vitro*, and to define the regulatory mechanisms involved.

Despite the cytokine family having a major impact, both physiologically and pathologically, upon cellular growth, metabolism and differentiated function very little data exists on their role in IGFBP regulation. In this study the fibroblast cell culture model has been used to clarify the stimulating effect of TGF $\beta$  upon IGFBP-3 (Martin & Baxter, 1991) and also to analyse for the first time the effect on the IGFBPs of the inflammatory cytokines TNF $\alpha$ , IL-1 $\beta$  and IL-6. As a function of these cytokines appears to be mediation of metalloproteinase production, notably in fibroblasts, their possible role in mediating IGFBP proteolytic activity was also investigated.

3. To develop a precise bioassay capable of measuring the effects of endogenous IGFBP regulation on IGF-I mitogenic activity.

The role that the IGFBPs play in mediating IGF activity at the cellular level is controversial. Based upon experiments using either purified or recombinant exogenous IGFBPs most *in vitro* data suggest that they are inhibitory, although they may also potentiate both the mitogenic and metabolic actions of the IGFs under certain incubation conditions. In an attempt to clarify this situation we have developed a precise mitogenic bioassay based upon the Hs68 fibroblast model described above to investigate the effect on IGF-I bioactivity of *endogenous* IGFBP regulation by TGF $\beta_1$  and TNF $\alpha$ . To distinguish those effects mediated via IGFBPs rather than membrane receptor or intracellular processes, comparison was made between the mitogenic activity of both native IGF-I and a synthetic IGF-I peptide with minimal affinity for the IGFBPs.



**CHAPTER 2.**  
**MATERIALS**

## **2.1. Peptides**

i. Human IGF-I (recombinant)	GroPep Pty Ltd., Adelaide, Australia. Kabi Pharmacia, Milton Keynes, Bucks, UK.
ii. LongR <sup>3</sup> IGF-I (recombinant)	GroPep Pty Ltd., Adelaide, Australia.
iii. Human IGFBP-3 (glycosylated and non-glycosylated, recombinant)	Celtrix Inc., La Jolla, California, USA.
iv. Human insulin (recombinant, 'Actrapid')	Calbiochem, Nottingham, UK.
v. Human TNF $\alpha$ (recombinant)	Bachem (UK) Ltd., Saffron Walden, Essex, UK.
vi. Human IL-1 $\beta$ (recombinant, code 86/680)	National Institute for Biological Standards and Control (NIBSC), Potters Bar, Herts., UK.
vii. Human IL-1 $\alpha$ (recombinant, code 86/632)	NIBSC, Potters Bar, Herts., UK.
viii. Human IL-6 (recombinant, code 88/514)	NIBSC, Potters Bar, Herts., UK.
ix. Human interferon gamma (recombinant)	Bachem (UK) Ltd., Saffron Walden, Essex, UK.
x. Human TGF $\beta$ <sub>1</sub> (purified, platelets)	British Biotechnology Ltd., Oxford, Oxon, UK.



xi. Prostaglandin E <sub>2</sub> (synthetic)	Sigma Chemical Co.Ltd., Poole, Dorset, UK.
xii. Bovine fibronectin (purified, plasma)	Sigma Chemical Co.Ltd., Poole, Dorset, UK.
xii. Gly-Arg-Gly-Asp fibronectin fragment (synthetic)	Sigma Chemical Co.Ltd., Poole, Dorset, UK.

## **2.2. Major Equipment**

### **Centrifuges**

Sorvall RT 6000b Benchtop	Du Pont (UK) Ltd, Wedgewood Way, Stevenage, Herts., UK.
MSE Coolspin	MSE Scientific Instruments, Manor Royal, Crawley, Sussex, UK.
MSE Centaur II	MSE Scientific Instruments, Manor Royal, Crawley, Sussex, UK.
MSE Microcentaur	MSE Scientific Instruments, Manor Royal, Crawley, Sussex, UK.
Beckman J-6B	Beckman Instruments, Beverley, California, USA

### **Power Supply Units**

LKB Bromma 2197	LKB Instruments Ltd, Selsdon, Croydon, Sussex, UK.
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Gamma Counter

NE 1600 Multihead

Nuclear Enterprises, Edinburgh,  
UK.

Gel Electrophoresis Tank

BRL V16-2

Bethesda Research Labs, Trident  
House, Renfrew Rd, Paisley, Scotland.

Transfer Unit

LKB 2005

LKB Instruments Ltd, Selsdon,  
Croydon, Sussex, UK.

Vacuum Dessicator

GyroVap 02GV2

VA Howe and Co., St. Anne's  
Crescent, London, UK.

pH Meter

EDT GP 353

Pentacourt Ltd, Halstead, Essex, UK.

Water Purification System

Milli Q

Waters Millipore, Harrow, Middlesex,  
UK.

Water Baths

Grant JB1 and SE10

Chemlab Instruments, Hornchurch,  
Essex, UK.

CO<sub>2</sub> Incubator

Gallenkampf Controlled CO<sub>2</sub>

Gallenkampf, Christopher St., London,  
UK.



Laminar Flow Cabinet

Envair MSC II

Envair (UK) Ltd, Rossendale, Lancs.,  
UK.

Balances

Mettler PM 300

Gallenkamp, Christopher St., London,  
UK.

Chan 28 Automatic Electrobalance

WT Avery Ltd, Smethwick,  
W.Midlands, UK.

Stanton MC9

Stanton Instruments Ltd, London, UK.

Microscope

Olympus CK

Olympus, Tokyo, Japan.

Temperature Controlled Incubator

Leec MkII

Luckhams, Burgess Hill, Sussex, UK.

Fraction Collector

Ultrorac 2070

LKB Instruments Ltd, Croydon, Sussex,  
UK.

Oil Pump

High Vacuum ED100

Edwards High Vac Ltd, Manor Royal,  
Crawley, Sussex, UK.

Gel Dryer

BioRad 543

BioRad Inc., Hemel Hempstead, Herts.,

UK.

### Peristaltic Pump

Chem Lab CPP 15

Chem Lab Instruments, Hornchurch,  
Essex, UK.

### Densitometers

Model 450 microplate reader with  
595nm and 650nm wavelength filters

BioRad Inc., Hemel Hempstead, Herts.,  
UK.

LKB Ultrascan XL scanning  
densitometer

Pharmacia LKB, Biotechnology,  
Uppsala, Sweden.

Shimadzu UV-150-02 spectrophotometer

Shimadzu Co., Kyoto, Japan.

## **2.3. Minor Equipment**

### Vortex Mixer

Hook & Tucker Instruments Ltd, New  
Addington, Croydon, UK.

### Magnetic Stirrer

Jencons Scientific Equipment, Hemel  
Hempstead, Herts.

### Gas

95% O<sub>2</sub> : 5% CO<sub>2</sub>

British Oxygen Company, London, UK.

### Pipettes



Finn Pipettes	Jencons Scientific Equipment, Hemel Hempstead, Herts, UK.
Eppendorf Pipettes	Anderman & Co., Kingston-on-Thames, Surrey, UK.
<u>Plastics</u>	
Pipette Tips Rainin S20 and S200	Anachem, Luton, Beds., UK.
Trieff Lab Microfuge Tubes	Scotlab, Bellshill, Strathclyde, UK.
Siliconized Microfuge Tubes	Sigma Chemical Co. Ltd, Poole, Dorset, UK.
Nunc Sterile 50 ml Centrifuge Tubes	Gibco, Paisley, Renfrewshire, UK.
RIA Tubes (2.5 ml)	Griffiths & Nielson Ltd, Billingshurst, Sussex, UK.
Nunc Tissue Culture Flasks	Gibco, Paisley, Renfrewshire, UK.
Cryostat Tubes	Sterilin Ltd, Alton, Hants., UK.
Sterile Pipettes (1, 5, 10 ml)	Sterilin Ltd, Alton, Hants., UK.

Sterile Syringes & Needles

Sterilin Ltd, Alton, Hants., UK.

### Glassware

Glassware, pipettes, measuring cylinders, volumetric flasks, electrophoresis plates, beakers, magnetic stirrers.

Jencons Scientific, Hemel Hempstead, Herts, UK.

Glass pasteur pipettes

John Paulten Ltd, Barking, Essex, UK.

Laboratory chromatography columns

Pharmacia, Milton Keynes, Beds., UK.

### Size exclusion gels

Sephadex G25 and G50

Pharmacia, Milton Keynes, Beds., UK.

Pharmacia 1.0 x 30 cm Superose 12

Pharmacia, Milton Keynes, Beds., UK.

AcA 202 size exclusion gel

Ultragel IBF, Biotechnics, Villeneuve, France.

### Autoradiography Film

Kodak XAR5 Film

Kodak, Hemel Hempstead, Herts., UK.

Amersham Hyperfilm MP

Amersham International plc.,



Amersham, Bucks.

Autoradiography Cassettes

Genetic Research Instruments, Bishops

Stortford, Herts., UK.

Membranes

Hybond C+

Amersham International plc.,

Amersham, Bucks. UK.

Biotrans

ICN Biomedical Inc., Irvine, CA. USA.

Filters

3mm paper

Whatman International Ltd, Maidstone,

Kent, UK.

Sterile 0.2  $\mu$ M filters

Schleicher & Schuell, Dassel, Germany.

Sterile Arodisc 0.2  $\mu$ M filters

Gelman Sciences, Michigan, USA.

**2.4. Reagents**

General laboratory chemicals (AnalaR or Molecular Biology Grade) and reagents for tissue culture were supplied by: BDH Chemicals Ltd, Poole, Dorset, UK., or Sigma Chemical Co. Ltd, Poole, Dorset, UK.

**2.5. Commercially available kits and preparations**

QIAGEN Plasmid Kit

QIAGEN Inc., Chatsworth, CA, USA.

GENECLEAN II

BIO 101 Inc., CA, USA

Oligolabelling kit

Pharmacia, Milton Keynes, Beds., UK.

RNAzol B

Biogenesis, Bournemouth, UK.



## **CHAPTER 3.**

### **METHODS**

**3.1. CELL CULTURE**

The fibroblast cells used throughout this thesis, designated Hs68, were obtained from the American Type Culture Collection (Rockville, Maryland, USA), number CRL 1635. They were first developed at the Naval Biosciences Laboratory (Oakland, California) and are derived from the foreskin of a normal, Caucasian newborn infant. Unspecified longevity studies carried out at source demonstrated that the cells could be successfully propagated for up to 40 passages. For our studies they were routinely used for experiments between passages 15 and 30 without noticeable alteration in morphology or IGFBP production.

**a. Cryopreservation**

Stocks of Hs68 cells (2-10 x 10<sup>6</sup> cells/cryotube) were kept frozen in liquid nitrogen stored in 95% fetal calf serum (v/v) plus 5% dimethyl sulphoxide (v/v). Resuscitation involved rapid thawing and transfer into pre-warmed 'growth medium', followed by centrifugation for 5 minutes at 50g, aspiration and seeding into tissue culture treated flasks in 'growth medium'.

**b. Growth medium**

Hs68 cells were routinely grown in a humidified incubator at 37°C, 5% CO<sub>2</sub> in the following medium:

Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose	
L-glutamine	2 mmols/l
Fetal bovine serum	10% v/v

Aspiration of spent medium and subsequent re-feeding was performed every 3-4 days.

**c. Trypsinisation procedure**

Growing adherant cell stocks were passaged every 7 days using the following solution:

Dulbecco's PBS;	
Sodium chloride	137 mmols/l
Sodium phosphate	95 mmols/l
Potassium chloride	2.7 mmols/l
Potassium phosphate	1.5 mmols/l



Trypsin (bovine)

0.5 g/l

EDTA

1 mmol/l

Spent growth medium was first aspirated, and then sufficient trypsinising solution added such that the monolayers were fully wetted. Following a brief 5 minute incubation at 37°C, the dispersed cells were placed into a sterile 30 ml universal tube and 10 mls of growth medium added to quench excessive trypsinising. The tube was then centrifuged (approximately 50g) for 5 minutes, the solution aspirated and the cells resuspended in 5-10 mls of growth medium for estimation of cell number using a haemocytometer. Flasks of new stock cultures ( $5 \times 10^4$ /ml) or experimental plates and dishes were subsequently seeded.

#### **d. Standard experimental conditions**

All cells committed for experiments were grown in growth medium for 3-4 days until approximately 80% confluent prior to placement into serum-free conditions. Essentially, this medium consisted of the growth medium described above but with 0.1% bovine serum albumin (BSA) replacing the serum component.

### **3.2. WESTERN LIGAND BLOTTING (WLB)**

Early analysis of IGFBPs mostly involved size separation gel chromatography followed by radiolabelled IGF binding studies. However, in addition to its laborious nature, the presence of saturating endogenous IGFs, particularly in serum samples, presented major difficulties. The development of a technique which overcame many of these problems was first described by Hossenlopp *et al* (1986). In this seminal paper, they demonstrated a methodology, named Western ligand blotting (WLB), that involved the separation of IGFBPs by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Crucially, the SDS incubation conditions were seen to *reversibly* dissociate endogenous IGFs from the binding proteins, allowing the binding of  $^{125}\text{I}$ -IGF following their transfer to nitrocellulose membranes. Visualisation of the IGFBPs, migrating at their molecular weights, could thus be achieved with autoradiography.

In this study we have utilised WLB ourselves for the determination of IGFBPs. It must be stressed however that this technique is not necessarily quantitative, as band intensities are determined by the combination of both the IGFBP's molecular mass *and* its binding affinities for the radiolabelled IGF ligand. With the discovery of certain proteolytic enzymes capable of altering IGFBP affinity this is especially pertinent to interpretation of WLB data, as will be seen in the following RESULTS section.

#### **a. Polyacrylamide gel electrophoresis (PAGE)**

Size separation of IGFBPs was performed by PAGE, as described by Laemmli (1970), using 12.5 % homogenous linear gradient acrylamide gel slabs under non-reducing conditions.

##### **Resolving Gel:**

acrylamide (30% w/v) +	
N,N'-Methylene Bis acrylamide (1% w/v)	27 ml
Tris base (1.5 mols/l, pH 8.9)	16.8 ml
Distilled H <sub>2</sub> O	15.6 ml
EDTA (0.1 mols/l, pH 7.4)	5.4 ml
SDS 10% w/v	2.7 ml



Ammonium persulphate (10% w/v)	0.6 ml
N,N,N,N-tetramethylethylenediamine (TEMED)	26 $\mu$ l

#### Stacking Gel:

30% w/v acrylamide + 1 % w/v Bis	3.75 ml
Tris-HCl (1.1 mols/l, pH 6.8)	1.125 ml
Distilled H <sub>2</sub> O	16.95 ml
EDTA (0.1 mols/l, pH 7.4)	0.45 ml
SDS (10%)	0.22 ml
Ammonium persulphate (10% w/v)	0.34 ml
TEMED	22.5 $\mu$ l

#### Electrophoresis Running Buffer, pH 9.6:

Tris base	6 g/l
Glycine	29 g/l
EDTA	1.5 g/l
SDS	2 g/l
Distilled H <sub>2</sub> O	1 l

#### Sample Buffer:

1.1 M Tris HCl pH 6.8	0.7 ml
Glycerol	1.1 ml
10 SDS	3.0 ml
Bromophenol blue (1 % solution)	0.2 ml

#### Method:

Two glass plates were first washed with 7X-PF detergent (ICN FLOW) and absolute methanol, then rinsed with distilled water. After assembly using 3mm spacers, the resolving gel was poured, overlaid with isopropanol to ensure a planar surface, and allowed to set for about 30 minutes. Once set, the isopropanol was poured off and approximately 2.5 cm of stacking gel added. The spacer comb was inserted and the gel

again left for 30 minutes to set. Once set, the comb was removed and wells flushed with distilled H<sub>2</sub>O ready for sample loading.

The samples (specific details of which are given in the relevant results chapters) were diluted with sample buffer and boiled for 5 minutes, before being cooled on ice. A maximum of 80µl of each sample was loaded into the lanes of the stacking gel. Pre-stained molecular weight markers (Life Technologies Ltd, Strathclyde, U.K.) were run in parallel lanes. Electrophoresis was performed at a constant current of 40 mA for 15 hours (70 mA for two gels).

### **b. Protein electroblotting of IGFBPs to nitrocellulose**

Two methods were used for transferring and immobilising the IGFBPs from the polyacrylamide gel to solid phase nitrocellulose membranes.

#### **i. Standard electroblotting**

Transfer Buffer;

Glycine	42.75 g
Tris-HCl	15.15 g
Distilled H <sub>2</sub> O	3 L

Following preparation of the above solution, a further 1L of MeOH, followed by 1L of distilled H<sub>2</sub>O was added, making a total volume of 5L. Finally, the pH was adjusted to 8.3.

Method:

Slab gels were removed from the glass plates, the stacking gel removed and discarded, and then placed on a sheet of 'Hybond-C extra' nitrocellulose (Amersham International, Bucks, U.K.). This was then sandwiched between two sheets of Whatman 3mm filter paper, followed by two pieces of foam padding, before being placed in the electro-transfer cassette. The cassette was placed into the electro-transfer tank (LKB 2005, Transphor unit), ensuring that its alignment allowed the proteins, moving from -ve to +ve, to transfer onto the nitrocellulose. Electroblotting was performed under a constant current, 0.8 - 1.0 Amps, for 4 hours at 4°C.



## ii. Semi-dry electroblotting

An alternative method of electroblotting without the requirement of large buffer volumes was also used. Firstly, four pieces of Whatman 3mm filter paper were cut to the same size as the gel and soaked in Transfer buffer as detailed above. A similar sized piece of nitrocellulose paper was then placed on top of the filter papers, followed by the gel itself, and a further four soaked filter papers. This sandwich was then placed onto a Semi-Phor electroblotter (Hoefer Scientific Instruments) and run at a constant current of 25 mAmps for 2 hours.

## c. Preparation of nitrocellulose for probing

Following electroblotting, the nitrocellulose sheet required treatment with a series of detergent washes and blocking of non-specific binding prior to incubation with <sup>125</sup>I-IGF.

### Solution #1, pH 7.4

Tris-HCl	0.8 g
Nonidet P-40 (NP40)	50 µl
Distilled H <sub>2</sub> O	100 ml

### Solution #2, pH 7.4

Tris-HCl	0.8 g
BSA	3.0 g
Distilled H <sub>2</sub> O	100 ml

### Solution #3, pH 7.4

Tris-HCl	0.8 g
Sodium phosphate	0.8 g
Tween 20	200 µl
Distilled H <sub>2</sub> O	100 ml

### Method:

The nitrocellulose filter was air dried for 15 minutes at 37°C, before being soaked in

solution #1 for 30 minutes at 37°C. This was followed by incubation with solution #2, for 90 minutes, and then solution #3, for 20 minutes, both at 37°C. The blot was then allowed to dry at room temperature.

#### **d. $^{125}\text{I}$ -IGF binding**

The IGFBP bands on the nitrocellulose were visualised by incubation with radiolabelled IGF-I. Iodination of recombinant human IGF-I was performed using the chloramine-T method. This technique relies upon  $\text{Na}^{125}\text{I}$  being oxidised by chloramine-T (N-chloro-p-toluenesulphonamide sodium salt) in the presence of the protein to be labelled, thus allowing the subsequent incorporation of  $^{125}\text{I}$  into the tyrosine residues of the protein. Excess oxidised  $\text{Na}^{125}\text{I}$  is quenched with a saturated solution of free tyrosine. After the addition of a protein containing buffer, to act as a carrier, the labelled IGF peptide is purified from the unincorporated iodide by size exclusion gel chromatography. The specific protocol was as follows:

Iodination reaction;

10  $\mu\text{g}$  IGF-I, in 10  $\mu\text{l}$  0.05 mols/l phosphate buffered saline (PBS)

1.0 mCi  $\text{Na}^{125}\text{I}$  (Amersham International, Bucks, UK) in 10  $\mu\text{l}$

1.25  $\mu\text{g}$  chloramine-T, in 5  $\mu\text{l}$  0.05 mols/l  $\text{NaH}_2\text{PO}_4$

The above were mixed together in a 1 ml centrifuge tube for 15-20 seconds at room temperature, and then mixed once more following the addition of;

5  $\mu\text{l}$  saturated tyrosine

and then by;

75  $\mu\text{l}$  0.05 mols/l PBS + 1% bovine serum albumin (BSA)

#### **Purification**

Sephadex G-25 was first pre-swollen overnight in 0.05 mols/l PBS + 1% BSA, packed into a 10 x 1 cm chromatography column and equilibrated with 50 ml of the same buffer. As the size separation of this gel is in the range 1000 to 5000 kDa, the radiolabelled IGF is fractionated in the initial void volume and is followed by the free  $^{125}\text{I}$ -tyrosine in later fractions. The fractions were collected every 45 seconds, giving volumes of approximately



0.25 ml, and the labelled peptide aliquoted and stored frozen at -20°C. A typical profile is shown in **figure 3.i.** overleaf.

Solutions were subsequently made for incubation with the nitrocellulose as follows and incubated overnight at 4°C.

Solution #4;

Tris-HCl	0.8 g
BSA	3.0 g
Distilled H <sub>2</sub> O	100 ml
<sup>125</sup> I-labelled IGF (60,000 counts/100µl/min)	

Solution #5;

Tris-HCl	0.8 g
Sodium phosphate	0.8 g
Tween 20	400 µl
Distilled H <sub>2</sub> O	100 ml

Solution #6;

as Solution #5, less Tween 20

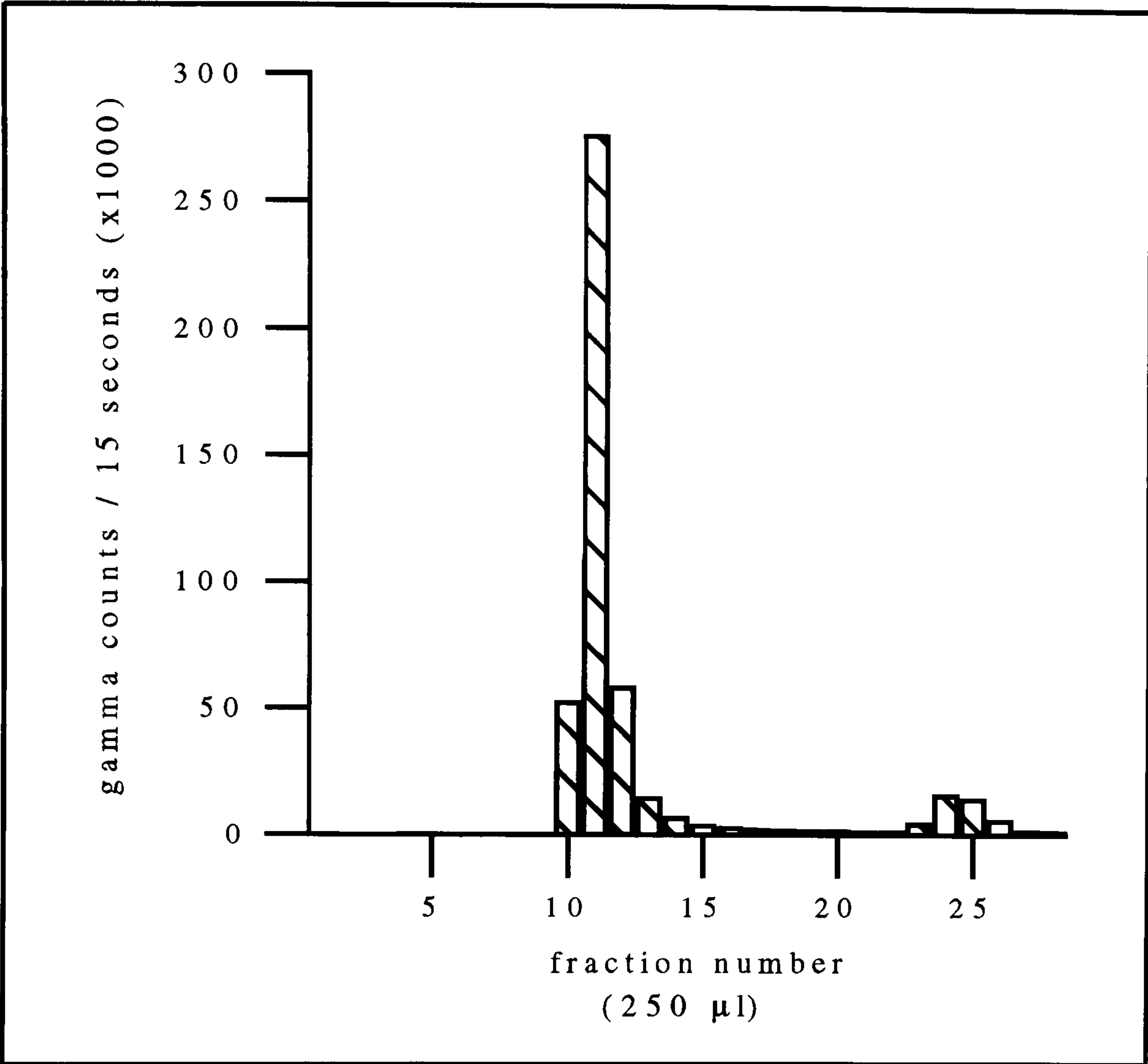
Method:

The nitrocellulose blot was first incubated with radiolabelled IGF-I, solution #4, for either 2 hours at room temperature or overnight at 4°C. It was then washed in solution #6 for 15 minutes at 4°C, and finally washed a further three times in solution #6. The blot was then air dried at room temperature.

**e. Autoradiography**

The dried blot was first covered with cling-film to avoid contaminating the photographic film. It was then exposed to X-ray film (Kodak X-OMAT RP, 20cm x 25.5cm) at -70°C for between 3 and 7 days, before being developed. Certain of the autoradiographs were quantitatively analysed by scanning densitometry.

Figure 3.i.



Sephadex G25 size exclusion chromatographic profile of IGF-I following radiolabelling with  $^{125}\text{I}$ , as described in section 3.2.d. Radiolabelled IGF-I eluted in fractions 10 to 15, with free tyrosine in fractions 24 to 25.



### **3.3. IGFBP-3 RADIOIMMUNOASSAY**

IGFBP-3 immunoreactivity in cell conditioned medium was determined by RIA, based upon that of Cwyfan-Hughes *et al* (1993) developed in this laboratory using recombinant glycosylated IGFBP-3 for standards and tracer (generously provided by Dr C. Maack, Celtrix, Santa Clara, CA, U.S.A.).

Assay buffer:

Sodium phosphate	4.68 g/l
Protamine sulphate	0.2 g/l
EDTA	3.72 g/l
Sodium azide	0.2 g/l

The buffer was titrated to pH 7.5 and stored at -20°C. Before use in assay, Tween 20 was added to a concentration of 0.05%.

#### **a. IGFBP-3 standards**

Assay standards were prepared from a stock of the CHO cell derived recombinant glycosylated IGFBP-3. The dilutions were made in a solution comprising 0.1% formic acid, 1% BSA and 0.9% NaCl, giving an analytical range of IGFBP-3 between 5 - 500 µg/l or 0.25 - 25 µg/tube. Standards were stored at -20°C until use.

#### **b. <sup>125</sup>I IGFBP-3 tracer**

Iodination of the peptide was performed using the chloramine-T method. This technique relies upon Na<sup>125</sup>I being oxidised by chloramine-T (N-chloro-p-toluenesulph onamide sodium salt) in the presence of the protein to be labelled, thus allowing the subsequent incorporation of [<sup>125</sup>I]iodine into the tyrosine residues of the protein. Excess chloramine-T is reduced by the addition of sodium metabisulphite, and the free iodine is reduced to iodide.

After the addition of a protein containing buffer, to act as a carrier, the labelled peptide is separated from the unincorporated iodide by gel filtration. The specific protocol adopted for iodination of IGFBP-3 was as follows:

Iodination reaction;

5 µg glycosylated IGFBP-3, in 10 µl H<sub>2</sub>O

0.5 mols/l sodium phosphate, 10 µl

0.5 mCi Na<sup>125</sup>I (Amersham International, Bucks, U.K.), 5 µl

5 µg chloramine-T, in 5 µl 0.05 mols/l sodium phosphate

The above were mixed together in a 1 ml siliconised tube for 15 - 20 seconds at room temperature, followed by the addition of;

5 µg sodium metabisulphate, in 5 µl 0.05 mols/l sodium phosphate

This reaction mixture was then mixed for a further 60 seconds at room temperature, before finally adding 75 µl of elution buffer, consisting of;

0.1 % formic acid + 0.5 % Tween 20 + 1 % BSA + 0.9 % NaCl

### Purification

Ultrogel AcA 202 (IBF, Villeneuve-la-Garenne, France) was packed into a 10 x 1 cm column. After being connected to a peristaltic pump, the gel was washed and equilibrated overnight with the above degassed elution buffer, at a flow rate of 3 ml/hour. As this particular gel has a separation range of between 1 and 15 kDa, once the iodination solution was applied to the column the radiolabelled IGFBP eluted in the void volume, that is in the early fraction numbers. The free <sup>125</sup>I, being able to fully enter the gel itself, eluted in the later fractions. Fractions were collected every 10 minutes, giving volumes of 0.5 ml, and were briefly counted in a gamma counter before being stored at -20°C. Specific activities were typically between 50 and 80 µCi/µg.

### c. Antisera

For the majority of the data described in this study a single rabbit polyclonal antiserum was used, designated 1287-2-14 from Celtrix. This antibody was found to be at optimal



titre at a dilution of 1:20,000 in assay buffer (Cwyfan-Hughes *et al*, 1993). Results obtained from 1287-2-14 were affected by the presence of the IGF-I peptide however, presumably because the epitope it recognised was near to the IGF binding site on the IGFBP-3 molecule. To overcome the problem of fluctuating IGF levels in the assay sample all assay tubes were loaded with 2 ng (40 µg/l) IGF-I.

Conditioned medium from our Hs68 line was found to dilute in parallel with the IGFBP-3 standards (**figure 3.ii.**), whilst proteolytic activity from these cells was without effect upon the assay (**figure 3.iii.**). This latter point was demonstrated by similar IGFBP-3 data obtained from samples incubated at 37°C for 24 hours in the presence or absence of a known inhibitory concentration (20 mmols/l) of EDTA.

In addition to 1287-2-14, two further IGFBP-3 rabbit antisera were also used for some of our studies. The first, SCH 2/5, was raised against the recombinant IGFBP-3 from Celtrix, and was used at a final dilution of 1:8000. Unlike antiserum 1287-2-14, its binding to <sup>125</sup>I IGFBP-3 was unaffected by the presence of IGF-I.

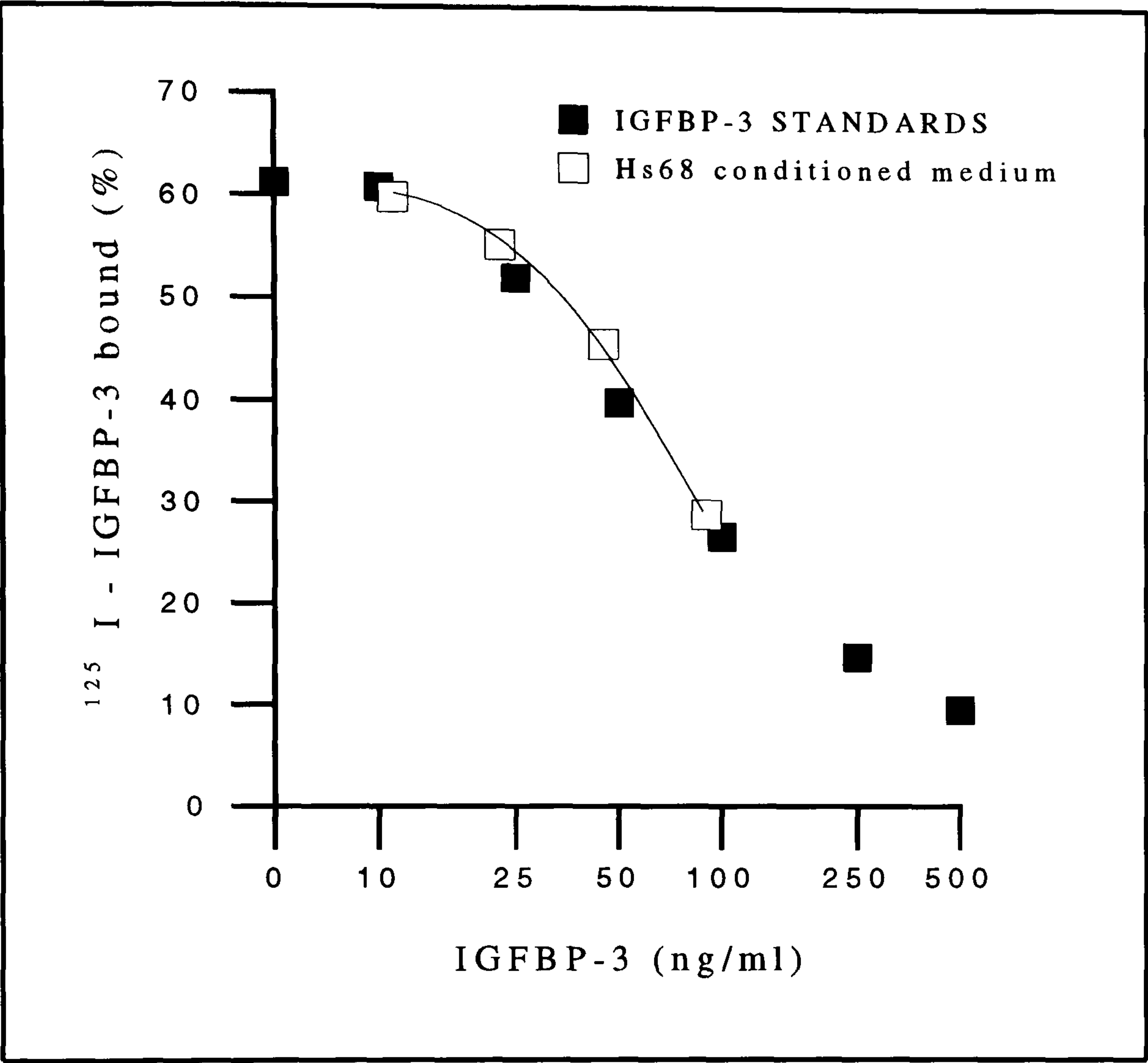
The second, α-BP-3gl, was provided by Dr R.G.Rosenfeld, and has been characterised at Stanford University, U.S.A., (Gargosky *et al*, 1992). It was used at a final dilution of 1:50,000 and again its binding to IGFBP-3 was not affected by the presence of IGF-I.

#### **d. Assay methodology**

A volume of 300 µl of assay buffer was first added to a 3 ml polystyrene assay tube. This was followed by 50 µl of the IGF-I solution (40 µg/l) used to overcome sample variation in this peptide. 50 µl of sample or IGFBP-3 standard was then added, followed by 50 µl <sup>125</sup>I IGFBP-3, diluted in assay buffer to give approximately 10,000 counts/minute. Finally, 50 µl of diluted antiserum was added (except to non-specific binding, NSB, tube) and the assay tube vortexed and incubated overnight at 4°C.

Separation of bound and free antigen was then achieved by adding for 30 minutes at 4°C a volume of 50 µl of donkey anti-rabbit IgG coated cellulose (Sac-Cel, IDS, U.K.). 1 ml of distilled H<sub>2</sub>O was then added to each tube, vortexed briefly, and centrifuged at 1000g for 30 minutes at 4°C. Supernatants were aspirated and the remaining pellets counted in a gamma counter (Nuclear Enterprises, Edinburgh, Scotland) for 100 seconds.

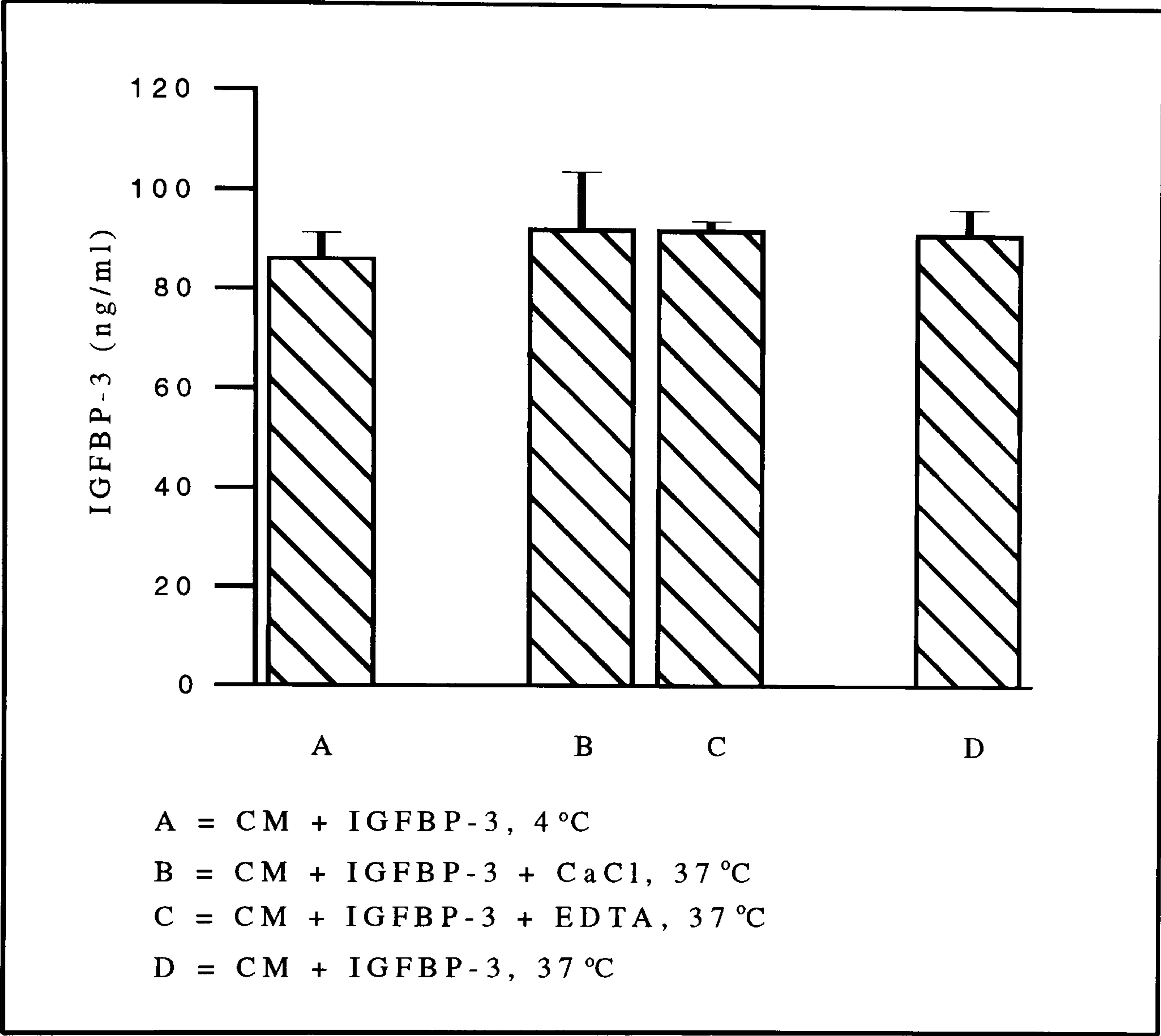
Figure 3.ii.



Serial dilution of Hs68 fibroblast conditioned medium in the IGFBP-3 radioimmunoassay (antiserum 1287-2-14) - comparison with glycosylated IGFBP-3 standards.



Figure 3.iii.



The effect of Hs68 cell conditioned medium (CM) protease on IGFBP-3 immunoreactivity, assessed by radioimmunoassay (antiserum 1287-2-14). 24 hour CM from untreated cells was loaded with 50 ng/ml exogenous IGFBP-3 and incubated for 24 hours as outlined above before quantitation. CaCl and EDTA were used at 20 mmols/l.

### **3.4. MOLECULAR BIOLOGY**

When analysing the regulation of the secretion of a peptide, such as an IGFBP, it is important to establish if the specific mechanism of the regulation occurs at the gene expression level or is post-transcriptional. As a number of IGFBPs, including IGFBP-3, have been seen to be both 'stored' on cell surfaces and degraded by proteolytic enzymes, the potential for such post-transcriptional regulation would seem high, making experiments investigating gene expression essential.

In this study we have utilised the Northern blotting technique to examine regulation of Hs68 fibroblast IGFBP-3 gene expression. Essentially, this consists of isolation of total RNA from cell monolayers, electrophoresis of denatured RNA through agarose gels, the transfer of fractionated RNA to nylon or cellulose membranes and finally the hybridisation of the immobilised RNA to highly specific radiolabelled probes. Visualisation of bands is made by autoradiography. Our particular protocol was as follows.

#### **a. DNA Probes**

##### **i. IGFBP-3 cDNA**

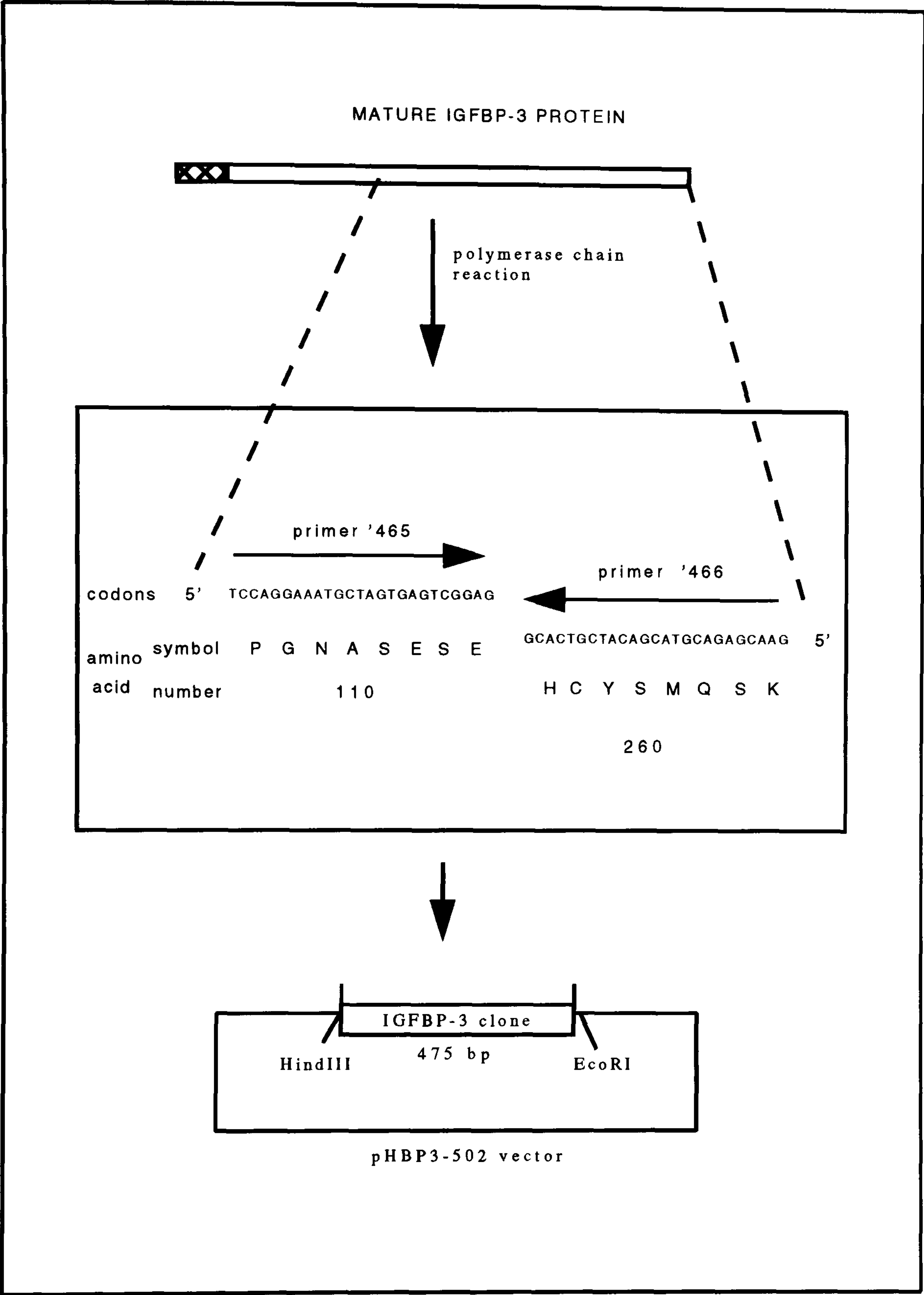
The human IGFBP-3 cDNA probe that was used throughout this thesis was a generous gift from Dr S.Shimasaki (1989). It consisted of a 475 bp fragment of IGFBP-3 DNA (see **figure 1.iii**), coding for approximately 60% of the mature IGFBP-3 protein, and was originally prepared by polymerase chain reaction and ligation into the pHBP3-502 plasmid vector, as shown in **figure 3.iv**.

##### **ii. IGFBP-4 cDNA**

A human IGFBP-4 cDNA was generously supplied by Dr D.R. Clemmons (University of North Carolina, NC, USA) having been prepared as previously described (Camacho-Hubner *et al* (1992)). Briefly, primers were designed to amplify by polymerase chain reaction a 462 bp segment of DNA that was located in the centre of the IGFBP-4 protein coding region. The amplified DNA was subsequently ligated into the EcoR1 site of the PBS vector that had been EcoR1 digested.



Figure 3.iv.



Schematic representation of human IGFBP-3 cDNA probe construction. The resultant pHBP3-502 plasmid was obtained from Dr.S.Shimasaki and treated as described in the Methods. Adapted from Shimasaki et al (1988).

## **ii. $\beta$ -Actin cDNA**

For some of the final Northern blot analyses described in this thesis a control cDNA probe was used to hybridise the Northern blot membranes, providing a precise record of the quantity of RNA loaded on the gels.  $\beta$ -actin is a widespread structural protein found throughout all eucaryotic cells and as such has been established as a useful control cDNA probe in a large number of studies. The cDNA used in this study was kindly supplied by Dr S.Chew, St.Bartholomew's Hospital, and encoded for a 689 bp section of the 1761 bp human  $\beta$ -actin mRNA. It was constructed by polymerase chain reaction using primers at bp position 144 and 833, and was labelled as described for the IGFBPs.

## **b. Transformation**

Competent *E.coli* cells (JM109) were transfected with either IGFBP-3 or IGFBP-4 plasmid. Firstly, to 50  $\mu$ l of cells approximately 10 ng plasmid (in a 1  $\mu$ l volume) was added and left on ice for 10 minutes. The solution was then heat shocked at 42°C for exactly 47 seconds before being placed on ice once more. An 800  $\mu$ l volume of LB broth (Sigma) was subsequently added and mixed with the reaction solution by gentle inversion before being incubated for 1 hour at 37°C. A 100  $\mu$ l volume of reaction solution was then incubated overnight at 37°C on culture plates prepared with sterile LB broth containing 1.5% (w/v) bacto agar and 100 mg/ml ampicillin. As IGFBP-3 and IGFBP-4 plasmids carried antibiotic resistance, this step allowed simple selection of those cells that had undergone successful transformation. A single colony of these cells was then cultured for 6 hours at 37°C in a 20 ml solution of LB broth + ampicillin (100 mg / ml). Finally, the 20 ml solution was mixed with 500 mls of LB broth and cultured overnight at 37°C.

## **c. IGFBP cDNA purification**

Plasmid DNA, containing the IGFBP cDNA, was initially purified from cultured *E.coli* cells using the QIAGEN Plasmid Kit (QIAGEN Inc., CA, USA). This method involves gently lysing the bacteria under alkaline conditions which results in the liberation of the plasmid without completely lysing the cell and releasing unwanted chromosomal DNA. RNA is removed by the action of exogenous RNAses. Separation of plasmid DNA from chromosomal DNA is achieved by the addition of acidic potassium acetate. This high salt



concentration causes the denatured proteins, chromosomal DNA, cellular debris and the SDS to precipitate, while the shorter plasmid DNA renatures correctly and stays in solution. The precipitate is then easily removed by high speed centrifugation, leaving a clear supernatant which can then be loaded onto the anion-exchange column provided in the kit. The DNA is finally eluted from the column by the addition of a specific salt concentration.

The detailed protocol was as follows:

#### **i. Cell resuspension**

Bacterial cells were first transferred from culture vessels to 50 ml sterile centrifuge tubes and separated from the culture broth by centrifugation at 4°C for 15 minutes at 6000 rpm (Sorvall GSA). The pellet was then fully resuspended in 10 mls of buffer designated **P1**, consisting of;

100 µg/ml RNase A

50 mM Tris / HCl

10 mM EDTA

pH 8.0

#### **ii. Cell lysis**

DNA and cellular protein was denatured by the addition of 10 ml of buffer designated **P2**;

200 mM sodium hydroxide

1% SDS

The above cell solution was gently mixed to ensure maximum denaturation by inverting the centrifuge tube briefly, and incubated at room temperature for 5 minutes. Vigorous mixing was avoided as this results in the shearing of chromosomal DNA, allowing its undesirable co-purification with the plasmid DNA.

#### **iii. Neutralisation and precipitation**

To the above lysate 10 ml of buffer **P3** was added, consisting of;

3 M Potassium acetate

pH 5.5

The solution was then thoroughly, but gently, mixed to ensure the maximum precipitation

of genomic DNA, cellular protein and especially the SDS. Failure to remove fully the SDS inhibits the ability of the plasmid DNA to bind to the purification column. The solution was incubated for 20 minutes on ice.

#### **iv. Column purification of plasmid DNA**

The 'QIAGEN-tip' anion exchange column was equilibrated with 10 ml of **QBT** buffer consisting of;

750 mM sodium chloride

50 mM MOPS

15% ethanol                      titrated to pH 7.0

The precipitated lysate from **step 3** above was transferred to ultracentrifuge tubes and spun at 30,000 g for 30 minutes at 4°C. The supernatant, hopefully containing mostly plasmid DNA, was then loaded onto the column at room temperature, followed by 2 washes of 30 ml of **QC** buffer consisting of;

1.0 M sodium chloride

50 mM MOPS

15% ethanol

pH 7.0

These wash steps effectively remove protein and carbohydrate contaminants from the DNA preparation.

The plasmid DNA is eluted from the column by the addition of 15 ml of buffer **QF** which consisted of;

1.25 M sodium chloride

50 mM Tris / HCl

15% ethanol

pH 8.5

DNA was then collected by precipitation with 10.5 ml (0.7 volumes) of isopropanol at room temperature and ultracentrifuged at 10,000 rpm or approximately 15,000 g for 30 minutes at 4°C. Following careful aspiration of the supernatant, the DNA pellet was



washed with 2 mls of cold 70% ethanol to remove any precipitated salt and replace the isopropanol with the more volatile ethanol, making the DNA easier to redissolve.

The pellet was subsequently briefly air dried and redissolved in 100 µl of sterile **TE** buffer comprising;

10 mM Tris / HCl

1 mM EDTA

pH 8.0

#### **v. DNA calculation**

The calculation of DNA in the above preparations were made using the following equation;

absorbance 260nm x K (constant) x dilution = µg DNA

For the 1:100 diluted IGFBP-3 preparation the absorbance value obtained was 0.067, giving the calculation as follows;

$$0.067 \times 50 \times 100 = 335 \text{ µg / ml}$$

equating to a total of 33.5 µg in 100 µl TE buffer

#### **vi. Isolation of IGFBP-3 cDNA**

Using information derived from the map provided in **figure.3.iv.** the IGFBP-3 cDNA was cut from the plasmid by the use of restriction enzymes EcoR1 and HIND III. The protocol was as follows;

30 µl (approximately 10 µg) IGFBP-3 containing plasmid

2.5 µl EcoR1

2.5 µl HIND III

5 µl NEB (2) buffer

10 µl H<sub>2</sub>O

The solution was vortexed and the reaction allowed to run for 1 hour at 37°C before being stopped by a further incubation at 65°C for 3 minutes.

Assessment of the efficiency of the above reaction and isolation of the cDNA of interest

was performed by size separating the sample through a mini (10 x 6 cm) 1 % (w/v) agarose gel, as described in the **electrophoresis** section of the Northern blotting RNA methodology. For these DNA experiments the running buffer was changed however to **TAE** buffer consisting of;

40 mM Tris base

1.14 ml glacial acetic acid

1 mM EDTA

1 L H<sub>2</sub>O

The IGFBP-3 cDNA sample was prepared as follows;

2 µl restriction enzyme treated IGFBP-3 cDNA plasmid

3 µl DNA loading dye

30% glycerol

0.25% xylene cyanol

0.25% bromophenol blue

69.5 H<sub>2</sub>O

5 µl H<sub>2</sub>O

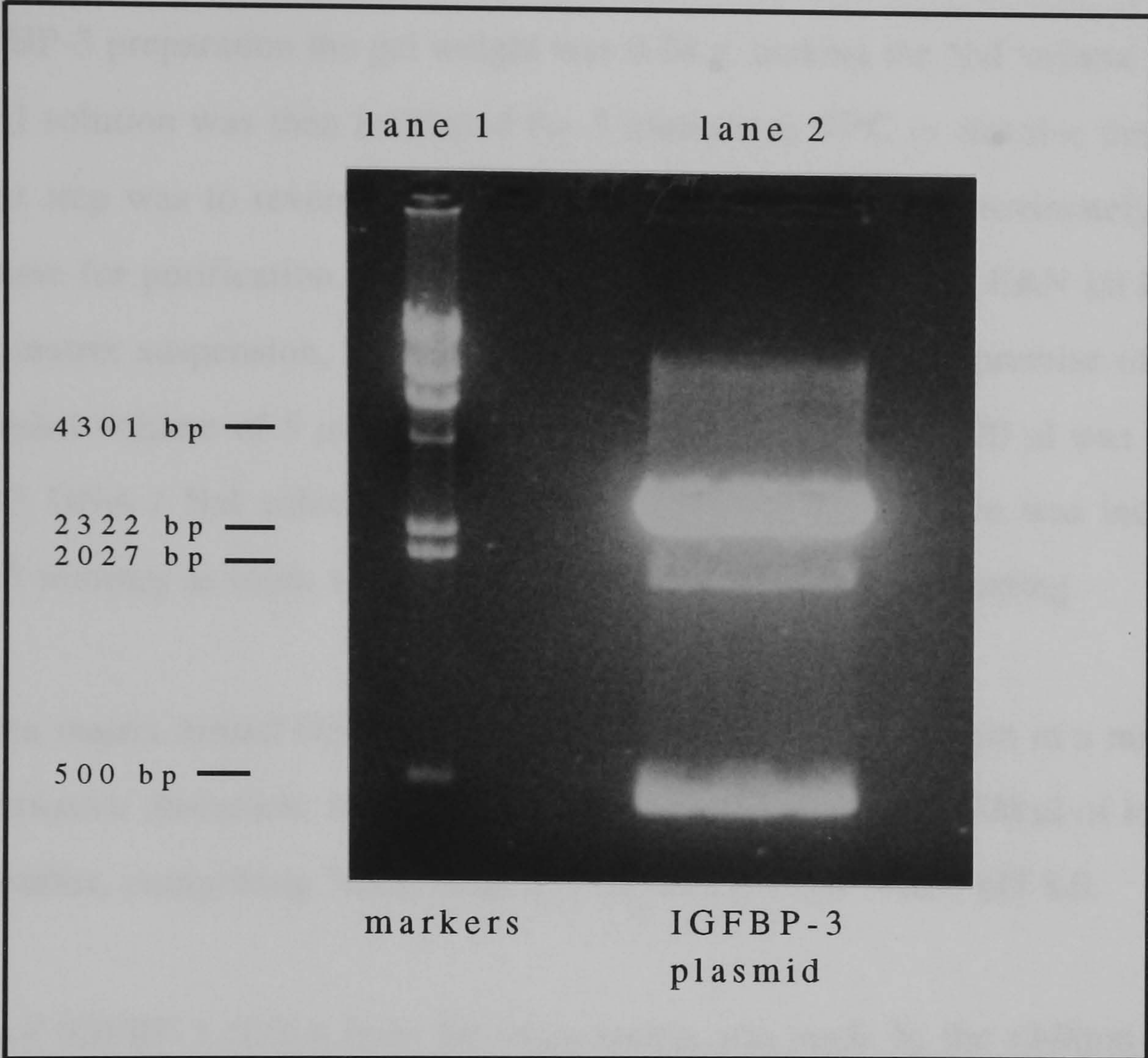
For estimation of DNA size a single (2 µl) sample containing a commercially available Hind III treated lambda phage DNA, with known molecular weight DNA fragments, was also included. The electrophoresis unit was ran for approximately 2 hours at 70 mAmps. With the aid of intercalated ethidium bromide the bands of DNA were subsequently revealed by transillumination of the gel under UV light and photographed. The results for IGFBP-3 are shown in **figure 3.v.** overleaf. Lane 1 shows the Hind III treated lambda phage DNA, whilst lane 2 shows the EcoR1 / Hind III treated IGFBP-3 cDNA plasmid, showing one major band migrating at an estimated weight of just below 500 bp.

#### vii. DNA purification from gel

Removal and purification of the IGFBP-3 cDNA band from the agarose gel seen in **figure 3.v.** was performed using the GENECLAN II kit (BIO 101 Inc.,CA,USA). The procedure



Figure 3.v.



DNA size assessment of the plasmid containing the IGFBP-3 cDNA clone following incubation with HIND III and EcoR1 restriction enzymes. Molecular weight markers from HIND III treated lambda phage are shown in Lane 1. The predicted size of the IGFBP-3 cDNA is 475 bp (Shimasaki et al,1988).



was as follows;

With the aid of UV light the band containing the 500 bp IGFBP-3 cDNA was excised from the gel using a sterile surgical scalpel. The weight of the gel slice was determined and it was then placed into a sterile 1.5 ml microfuge tube. On the assumption that 1 g is the equivalent of 1 ml of gel, 3 volumes of a 6 M sodium iodide (NaI) was added. For our IGFBP-3 preparation the gel weight was 0.24 g, making the NaI volume 0.75 ml. The gel / NaI solution was then incubated for 5 minutes at 50°C to dissolve the agarose.

The next step was to reversibly attach the solubilised DNA (approximately 10 µg) to a solid phase for purification. This was achieved with the GENECLAN kit by the use of a silica matrix suspension, known as 'Glassmilk'. Based on the premise of a minimum recommended volume of 5 µl Glassmilk for 5 µg DNA, a total of 20 µl was added to our IGFBP-3 DNA / NaI solution. After thorough vortexing, the tube was incubated for a further 5 minutes at room temperature to allow maximal DNA binding.

The silica matrix bound DNA was pelleted by a brief 5 second spin in a microfuge, and the supernatant discarded. It was then washed 3 times each with 500 µl of ice cold 'New Wash' buffer, comprising NaCl, Tris, EDTA, ethanol and water, pH 8.0.

Elution of IGFBP-3 cDNA from the silica matrix was made by the addition of a low salt solution. Thus, 10 µl sterile water was added to the solution, then vortexed and incubated for 3 minutes at 50°C. Following a 5 second microfugation to pellet the matrix, the supernatant, containing the DNA was carefully removed to a new sterile tube and the process repeated. The final volume was therefore 20 µl.

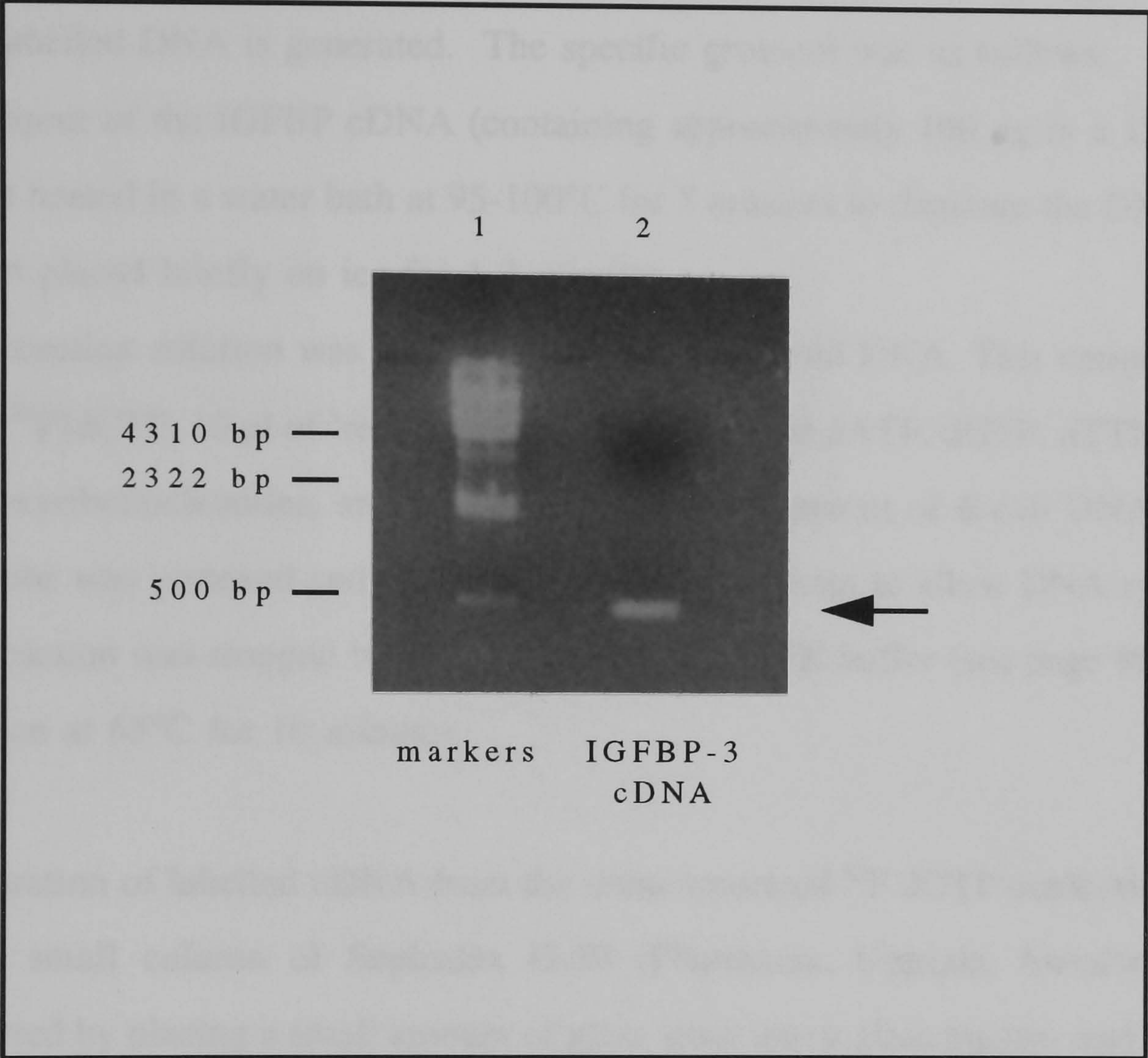
A final check on the integrity of the IGFBP-3 cDNA was made by running 2 µl of this solution on a 1 % agarose gel as previously described. Results of this are shown in **figure 3.vi. overleaf.**

#### **d. <sup>32</sup>P labelling of cDNA**

Labelling of IGFBP-3 and IGFBP-4 cDNA for use as hybridisation probes with the



Figure 3.vi.



Photograph of gel showing in lane 2 the size and integrity of IGFBP-3 cDNA (indicated by arrow) following purification from plasmid DNA with the GENE CLEAN kit. Standard molecular weight markers from HIND III treated phage DNA are shown in lane 1.



Northern blots was made with the 'Oligolabelling kit' supplied by Pharmacia Biotech. The IGFBP-3 cDNA to be labelled is first denatured and then mixed with hexadeoxyribonucleotides of random sequence. These random hexamers anneal to random sites on the DNA (now single stranded) and then serve as primers for new DNA synthesis by a polymerase. With the labelled nucleotide  $^{32}\text{P}$ -dCTP present during this synthesis, highly labelled DNA is generated. The specific protocol was as follows;

- i. An aliquot of the IGFBP cDNA (containing approximately 100 ng in a 15  $\mu\text{l}$  volume) was first heated in a water bath at 95-100°C for 5 minutes to denature the DNA. The tube was then placed briefly on ice for 1-2 minutes.
- ii. The reaction solution was then added to the denatured DNA. This comprised 25  $\mu\text{Ci}$  (25  $\mu\text{l}$ ) [ $^{32}\text{P}$ ]dCTP, 10  $\mu\text{l}$  of 'reagent mix' containing cold dATP, dGTP, dTTP and random hexadeoxyribonucleotides, and 1  $\mu\text{l}$  of the Klenow fragment of *E.coli* DNA polymerase I. The tube was vortexed and incubated at 37°C for 1 hour to allow DNA synthesis. The above reaction was stopped by the addition of 100  $\mu\text{l}$  TE buffer (see page 86) and a final incubation at 65°C for 10 minutes.

iii. Separation of labelled cDNA from the unincorporated  $^{32}\text{P}$  dCTP nucleotide was made using a small column of Sephadex G-50 (Pharmacia, Uppsala, Sweden). This was constructed by placing a small amount of glass wool into a glass pipette, and then packing approximately 3 mls of pre-swollen G-50 gel such that it left loading space for the sample and elution buffer.

Initially, the gel was washed with approximately 5 mls of TE buffer, and then loaded with the 150  $\mu\text{l}$  radiolabelled DNA sample. This was allowed to fully enter the column and eluted by successive washes with 200  $\mu\text{l}$  TE buffer. Fractions were collected and quantitated in a gamma counter. A typical profile is shown overleaf in **figure 3.vii**.

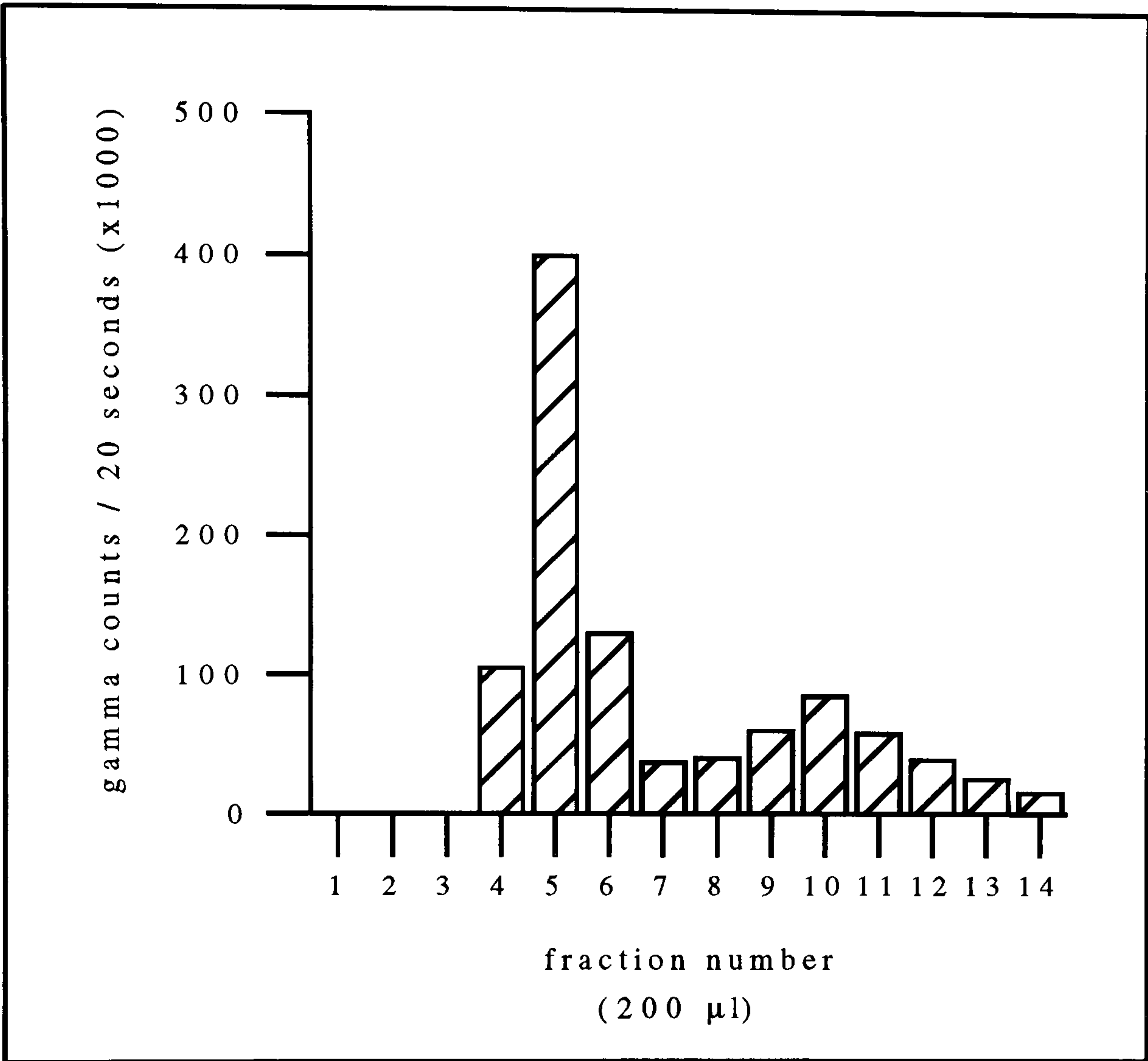
#### **d. Northern blotting**

##### **i. Isolation of Hs68 fibroblast RNA**

Extraction of RNA, based upon the guanidinium thiocyanate method (Chomczynski and Sacchi, 1987) was performed using RNazol B (Biogenesis, Bournemouth, U.K.). This solution promotes the formation of complexes of RNA with guanidinium and water, whilst



Figure 3.vii.



Size exclusion chromatographic profile of IGFBP-3 cDNA following radiolabelling with  $^{32}\text{P}$ -dCTP using the 'Oligolabelling kit' as described in section 3.4.c. The first peak corresponds to the cDNA, and the second to the unincorporated  $^{32}\text{P}$  nucleotides.

abolishing the hydrophilic interaction of DNA and proteins. The net result is that separation of the RNA, in the aqueous phase, can be made from the DNA and protein which can be pelleted and removed.

#### Cell homogenisation

Cell monolayers were first washed briefly with chilled sterile PBS (calcium and magnesium free) pH 7.4. To each culture dish 1 ml of RNazol B solution was added and the cells homogenised with a cell scraper. The resultant lysate was transferred to a sterile centrifuge tube.

#### RNA extraction

100 µl of chloroform was added to the homogenised cells and mixed thoroughly. The tube was then allowed to stand for a few minutes on ice and then microfuged at 13,000 rpm for 15 minutes at 4°C. The aqueous phase was subsequently removed and placed into a new sterile centrifuge tube.

#### RNA precipitation

An equal volume of isopropanol was added to precipitate the RNA in the aqueous phase. The tube was then allowed to stand for 15 minutes at 4°C (or overnight at -20°C) and the RNA pelleted by a further 30 minute 13,000 rpm spin at 4°C in a microfuge.

#### RNA wash

Following the above spin, the isopropanol was gently aspirated and a 200 µl volume of 70% EtOH added for a simple wash procedure. After vortexing the tube and another spin for 5 minutes at 13,000 rpm and 4°C, the ethanol was carefully aspirated and allowed to briefly air dry.

#### Calculation of RNA content

The dried RNA pellet was resuspended in 100 µl H<sub>2</sub>O and solubilised. A 1:100 dilution of the resultant solution was made in water and absorbances measured at 260nm and 280nm wavelengths in a spectrophotometer. For clean preparations of RNA the



absorbance ratio between 260nm and 280nm should be greater than 1.85.

Typical absorbance data obtained from a set of five Hs68 cell samples, each comprising pooled lysates from three culture dishes, is given below in **Table 2**:

**Table 2.**

Sample	Absorbance 260 nm	Absorbance 280 nm	Ratio 260:280nm	Total RNA (µg/ml)*
Control	0.030	0.015	2.00	120
IGF-I	0.037	0.019	1.95	148
LR3-IGF-I	0.051	0.026	1.96	208
Insulin	0.049	0.025	1.96	192
TGFβ1	0.045	0.023	1.96	168

\*Total RNA was calculated from :

$$\text{Absorbance 260 nm} \times 100 \text{ (dilution)} \times 40 \text{ (Constant)} = \mu\text{g/ml}$$

Following the calculation of RNA content, the RNA was precipitated once again with a volume of sodium acetate (2 mols/l, pH 5) sufficient to give a 10% (v/v) concentration, and 2.5 volumes of absolute ethanol. These samples were then stored at -70°C.

**ii. Electrophoresis**

**Denaturing the RNA**

Stored precipitated RNA samples were first thawed and microfuged at 13,000 rpm for 15 minutes at 4°C. The supernatant was aspirated and 50 µl absolute ethanol added to each tube, and the samples microfuged as before. The ethanol was then aspirated and the RNA allowed to briefly air dry. For the purpose of denaturing the RNA to allow electrophoretic size separation, each 15 µg sample was mixed with 12 µl of 'denaturing buffer' and incubated at 55°C for 60 minutes.

#### Denaturing buffer:

Glyoxal (deionised)	20% (v/v)
Dimethyl sulphoxide	50% (v/v)
H <sub>2</sub> O (sterile, DEPC treated)	20% (v/v)
Sodium phosphate (0.2 mols/l)	10% (v/v)

Following the above incubation, the denatured RNA samples were cooled on ice and mixed with 3 µl of gel-loading buffer, consisting of:

Sucrose (50% solution)	100 µl
Xylene cyanol (1% solution)	12.5 µl
Bromophenol blue (1% solution)	12.5 µl

The total sample volume of 15 µl was then loaded into each lane of the agarose gel, as described below.

#### Agarose gel electrophoresis

A 1% agarose solution was made up in sodium phosphate buffer (10 mmols/l). To fully solubilise the agarose it was necessary to first heat the solution fairly vigorously. Whilst the solution was cooling, ethidium bromide was then added to give a concentration of 0.005% (v/v). Once reaching a 'hand hot' temperature the agarose was poured into a taped-closed 14cm x 10cm horizontal gel block, all air bubbles removed and the spacer comb placed in position. The gel was then allowed to set for approximately 30 minutes at room temperature.

Once this was achieved the spacer comb and tapes were removed and the gel placed into the electrophoresis tank submerged in running buffer (10 mmols/l sodium phosphate, pH 6.8). RNA samples were then loaded into each lane, and the electrophoresis unit ran for 5 minutes at high power (120v) to quickly move the samples into the gel, simply to avoid their dispersion from the wells into the running buffer. The power was subsequently reduced to 70v, and recirculation of the buffer at 100 mls/hour began. After 3 - 4 hours at room temperature, the gel was removed, checked and photographed under ultra violet



light for assessment of loading in each lane.

Transfer of RNA

Following electrophoresis, the RNA was transferred to a nylon membrane (Biotrans, ICN Flow, U.K.) by capillary action. First, a piece of Whatman 3MM filter paper was placed on the over-turned electrophoresis block with its length sufficient to overhang the ends. This was then put into a large plastic box, and 20x sterile sodium citrate (SSC) buffer added.

20x SSC buffer:

Sodium chloride	175.3 g
Sodium citrate	88.2 g
H <sub>2</sub> O	1 l

titrated to pH. 7.4.

Once the filter paper was thoroughly wet and all air bubbles removed, the agarose gel containing the RNA was carefully placed on top, followed by the wetted nylon membrane cut to the exact size of the gel. To avoid 'short-circuits' and uneven transfer, the gel was surrounded by Parafilm. Subsequently, three pieces of wetted filter paper were then placed onto the nylon membrane and a 15cm high stack of paper towels, also cut like the filter paper to the size of the gel, finally added. A glass plate and a heavy weight were then placed onto the paper and the transfer allowed to run overnight at room temperature.

Due to the presence of intercalated ethidium bromide in the RNA, the gel and nylon membrane were then briefly checked to see if the transfer had been successful. Under ultraviolet light no RNA was seen in the gel, whilst the 18 S ribosomal RNA band just visible on the membrane. To fix the RNA to the membrane, it was subsequently air dried and baked in a vacuum oven for 2 hours at 80°C.

iii. cDNA hybridisation

The nylon membrane containing the RNA was first incubated for 12 hours at 55°C with a prehybridisation buffer, essentially to block non-specific binding of the radiolabelled

probe.

Prehybridisation / Hybridisation buffer:

Sodium phosphate (pH 7.2)	0.25 mols/l
EDTA	1 mmols/l
SDS	0.25 mols/l
Herring sperm DNA (denatured)	0.2 g/l

The radiolabelled IGFBP-3 cDNA was then diluted into 15 mls of the same buffer but without the herring sperm DNA, and incubated with the membrane for a further 24 hours at 55°C. Following this incubation, the membrane was washed in 2X SSC buffer + 3.5 mmols/l SDS first for 15 minutes at room temperature, and then in 1X SSC + 3.5 mmols/l SDS for a further 15 minutes at 50°C. Finally, it was washed in 0.1X SSC buffer + 3.5 mmols/l SDS for 1 hour at 65°C.

Autoradiography of the dried blot was performed at -80°C for between 3 and 5 days, and quantitation of the IGFBP-3 mRNA band was made using scanning densitometry.

#### **iv. Rehybridisation**

IGFBP-4 and  $\beta$ -actin hybridisation was performed on Northern blot membranes following initial IGFBP-3 analysis. Membranes were first subjected to a stringent wash procedure of 30 minutes at 60°C using a rehybridisation buffer:

Sodium phosphate (10 mM, pH 6.8)	50% v/v
Formamide	50% v/v

Subsequent hybridisation conditions were the same as described for IGFBP-3 above.



### **3.5. IGFBP PROTEASE ANALYSIS**

As outlined in the Introduction, the presence of proteolytic activity directed against the IGFBPs has now been described in pregnancy serum and in a number of pathological states. Their presence affects the binding affinity of the IGFBPs, particularly IGFBP-3, for radiolabelled IGF and has therefore been given as an explanation for the low IGFBP values in the serum of these patients when analysed by Western ligand blotting (WLB). Initial analysis of such activity was undertaken by incubating the sample of interest with a constant volume of normal serum (with little or no IGFBP protease activity) and subsequently assessing the IGFBP degradation by WLB (Giudice *et al*, 1990). Whilst this technique had certain advantages in that it was possible to assess the effect of the protease(s) on all IGFBP species found in normal serum, it also introduced into the incubation sample another source of proteases and associated inhibitors.

In this thesis an alternative technique first described by Lamson *et al* (1991) has been used which avoids the above problem. Rather than using serum IGFBPs followed by WLB, recombinant IGFBP-3 radiolabelled with  $^{125}\text{I}$  has instead been used as substrate. Visualisation of proteolytic activity is achieved by SDS polyacrylamide gel electrophoresis and autoradiography. The specific protocol was as follows.

#### **a. $^{125}\text{I}$ -IGFBP-3 substrate**

IGFBP protease activity in fibroblast conditioned media was analysed using radiolabelled human non-glycosylated IGFBP-3 (Celtrix, USA). This recombinant peptide is the 28 kDa core protein as expressed by *E.coli* cells and was labelled using the chloramine-T methodology exactly as described for the glycosylated IGFBP-3 in the Radioimmunoassay section of this Methods chapter. Separation of the  $^{125}\text{I}$ -IGFBP-3 from free unincorporated  $^{125}\text{I}$  was made by size exclusion chromatography using a 10 cm ACA 202 gel (IBF, Villeneuve-la-Garenne, France) as described previously. Typical specific activity was approximately 0.1 mCi/ $\mu\text{g}$  IGFBP-3.

#### **b. Digestions**

To a 1.5 ml siliconised centrifuge tube 90  $\mu\text{l}$  of CM from Hs68 fibroblast cultures, or unconditioned medium for control, was mixed with 2 - 4  $\mu\text{l}$  of  $^{125}\text{I}$ -IGFBP-3, giving a final

activity in the solution of approximately 10,000 counts / minute. To avoid any possible alterations in calcium ions in the CM sample which may alter the activity of the putative IGFBP metalloproteinase(s) 4 µl of 500 mmols/l CaCl<sub>2</sub>, giving a final concentration of 20 mmols/l, was also added. Samples were then incubated at 37°C for 3 - 24 hours

### c. Electrophoresis

Degradation of the <sup>125</sup>I-IGFBP-3 substrate was analysed by SDS Page, using reagents and buffers as described in the Western ligand blotting section of this Methods chapter. However, to aid later drying of the gel its thickness was reduced to 1.5 mm from the usual 3 mm, and the acrylamide content reduced from 12.5% to 11% to aid separation of possible IGFBP-3 fragments. Accordingly, gels were comprised of the following.

#### Resolving gel:

30% acrylamide + 1% Bis solution	16 ml
1.5 mols/l Tris base (pH 8.9)	11.2 ml
0.1 mols/l EDTA (pH 7.4)	3.6 ml
10% SDS	1.8 ml
distilled water	11.9 ml
10% APS	0.45 ml
TEMED	20 µl

#### Stacking gel:

30% acrylamide + 1% Bis solution	3.75 ml
1.5 mols/l Tris / HCl (pH 6.9)	1.125 ml
0.1 mols/l EDTA (pH 7.4)	0.45 ml
10% SDS	0.22 ml
distilled water	16.95 ml
10% APS	0.34 ml
TEMED	22.5 µl

Once the gels were constructed 20 µl of incubated sample was mixed with 40 µl of sample buffer, and the proteins denatured by placing them into boiling water for 5 minutes. 10



µl molecular weight rainbow markers (Amersham International, Bucks, UK) were diluted 1:7 with sample buffer and treated in a similar manner. The samples were then cooled and 30 µl loaded into each lane. Electrophoresis was performed at a constant current of 40 mA for approximately 4 hours.

#### **d. Autoradiography**

After electrophoresis the gel was taken from the electrophoresis unit and one of the glass plates carefully removed. The gel was lifted from the remaining plate by placing it onto a similar sized piece of Whatman 3mm filter paper and then transferred with the gel facing upwards onto a vacuum slab drier (Bio-Rad, Herts, UK). The gel surface was wetted with distilled water and then covered with cellophane membrane backing (Bio-Rad, Herts, UK) ensuring all air bubbles were removed by further wetting. The gel was subsequently dried under vacuum at 70°C for 90 minutes, and then exposed to X-ray film (Kodak X-OMAT RP) at -80°C for 2-3 days.

### **3.6. FAST PHASE LIQUID CHROMATOGRAPHY (FPLC)**

For size estimation of the  $^{125}\text{I}$ -IGFBP-3 substrate and its possible proteolytic fragmentation in the absence of the harsh biochemical conditions used in the electrophoretic analysis described above, neutral FPLC was also performed.

For these experiments a 1 x 30 cm pre-packed column of cross-linked agarose with a molecular weight separation range of between 1000 and 300,000 kDa (Superose 12) was used. It was first equilibrated at a flow rate of 0.5 ml/minute with 2 volumes (approximately 75 mls) of degassed and filtered (0.45  $\mu\text{m}$ ) running buffer:

#### FPLC run buffer

Sodium phosphate	50 mmols/l
NaCl	6 g/l
Sodium azide	100 mg/l
Tween 20	500 $\mu\text{l/l}$
pH 7.4	

100  $\mu\text{l}$  filtered (0.22  $\mu\text{m}$ ) samples containing the radiolabelled IGFBP-3 peptide were subsequently loaded onto the column using a glass Hamilton syringe and a 200  $\mu\text{l}$  HPLC loading loop. Fractions were collected every 30 seconds, giving fraction volumes of approximately 250  $\mu\text{l}$ , and assessed for radioactivity with a gamma counter.

Due to the unacceptable loss of the non-glycosylated IGFBP-3 peptide on the column, presumably a result of its high non-specific adherant properties as described by the manufacturers and Conover (1991), it was necessary to use the glycosylated variant in these studies. Its radiolabelling, purification and storage was identical to that described in the Radioimmunoassay section of this chapter.

As the predicted molecular weight of the glycosylated IGFBP-3 peptide has been given by the manufacturers as 40 kDa, the column was calibrated by running through 1 mg quantities (in 100  $\mu\text{l}$ ) of unlabelled bovine insulin (5.5 kDa) and ovalbumin (44 kDa) followed by analysis of fraction peak distribution by spectrophotometry at a 280 nm wavelength.



### **3.7. IGF BINDING STUDIES**

In addition to those IGFBPs secreted into the surrounding culture medium, there is increasing evidence that a number of IGFBPs associate *in vitro* with fibroblast cell surfaces and extracellular matrix components. Indeed, a study by Clemmons *et al* (1986) has revealed that up to 90% of IGF-I binding sites on these cells may comprise such immobilised IGFBPs, thus forming an important store of binding protein with a high potential for modulation of IGF bioactivity. We have therefore performed <sup>125</sup>I-IGF-I binding studies on Hs68 fibroblast monolayers (adapted from those described by Clemmons *et al*, 1991) to assess possible regulation of these IGFBPs.

#### **a. Cell culture**

Hs68 fibroblasts were initially seeded into 24-well tissue culture plates at a density of  $5 \times 10^4$  cells / cm<sup>2</sup> and in a volume of 0.5 ml / well DMEM + FCS, as described earlier. Following a three day incubation at 37°C the monolayers were placed into serum-free medium for a further 24 hours. Cells were then rinsed with fresh serum-free medium, and test samples added for another 24 hours before commitment to the binding assay.

#### **b. <sup>125</sup>I-IGF-I binding assay**

##### **i. Total IGF-I binding**

Assays were first performed to assess the binding of <sup>125</sup>I-IGF-I to all possible components on the cell surface; Type I IGF receptors, insulin receptors, and cell-associated IGFBPs. This was designated 'total binding'. Type II IGF receptors were not considered to be included in this group as very weak binding has only been observed with purified IGF-I (possibly contaminated with IGF-II) whilst no significant binding has been seen with recombinant IGF-I (Rosenfeld *et al*, 1987), the peptide used in our study.

Cells were first gently washed twice with ice cold phosphate buffered saline (PBS, 0.05 mols/l, pH 7.4) to remove IGFBPs in the culture medium, and then incubated for 2.5 hours at 10°C (to avoid active cellular uptake of the tracer) with 250 µl of assay buffer.

Assay buffer;

Minimum Essential Medium (MEM)	250 µl
Bovine serum albumin (BSA)	0.1% w/v

HEPES	20 mmols/l
<sup>125</sup> I-IGF-I	75,000 cpm
pH 7.4	

plus unlabelled IGF-I peptide over a concentration range of 0.1 - 100 nmols/l.

At the end of the incubation period the above assay buffer was aspirated and the cells washed twice with ice cold PBS as before. Following subsequent solubilization with sodium hydroxide (0.3 mols/l, 250 µl / well), the radiolabelled IGF-I content of each monolayer was assessed with a gamma spectrometer. Results are expressed as counts per minute of duplicate wells.

#### ii. Binding to IGFBPs

To determine the level and possible modulation of the cell-associated IGFBPs, similar experiments to those above were performed in the presence of 10 µmols/l unlabelled insulin which effectively abolishes binding of the <sup>125</sup>I-IGF-I to both type I IGF and insulin cell membrane receptors.



### **3.8. IGF BIOASSAY**

#### **a. Cytochemical bioassays**

The use of cytochemical techniques for the quantitative measurement of hormone bioactivity was first developed by Chayen and Bitensky (1968). These early *in vitro* bioassays involved the exposure of target tissue segments to the chosen hormone followed by measurement of certain cytochemical reaction products. After precipitation within the cell these highly coloured products are then analysed by scanning and integrating densitometry. Such bioassays were developed for adrenocorticotrophin (ACTH), thyroid stimulating hormone (TSH), gastrin and luteinising hormone (LH).

#### **b. Tetrazolium reactions**

Various substrates have been utilised in the development of this type of bioassay, such as the the production of ferric ferrocyanide (Prussian blue) from ferricyanide in the ACTH assay for example (Alaghband-Zadeh *et al*, 1974). However, perhaps the most widely used set of cytochemical reactions have involved the large group of tetrazolium salts. These soluble organic compounds are able to serve as indicators of biological reducing systems since they are converted into highly coloured insoluble formazans upon mild reduction (Altman, 1974). For the purposes of quantifying dehydrogenase activity, the formazan can be easily measured at its absorbance maximum using a spectrophotometer. In particular, the mono-tetrazole 3-[4,5-dimethyl thiazolyl-2]-2,4-diphenyl tetrazolium bromide (MTT) has been used in the study of cellular dehydrogenase activity, and was chosen for the IGF-I bioassay described in this study.

Essentially, dehydrogenase enzymes catalyze the transfer in certain metabolic pathways of hydrogen (or electrons) from a substrate which becomes oxidised, to an acceptor molecule which is then reduced. These reduced acceptors may be an enzyme prosthetic group, such as flavin adenine dinucleotide (FAD) which becomes FADH, or a co-enzyme, such as nicotinamide adenine dinucleotide / phosphate which become NADH and NADPH, respectively. They are then reoxidised via a number of intracellular hydrogen transport pathways which include the mitochondrial respiratory chain, the mitochondrial  $b_5$  system, and the cytochrome  $P_{450}$  system (Altman, 1974). The tetrazolium salt MTT can intercept the passage of the hydrogen ions along these transport chains, become reduced



and thus form its coloured formazan product. MTT has particular advantages over other tetrazolium salts, in that it is the most easily reduced, perhaps partly due to its low molecular weight (414 daltons) which allows it easier access across cell and subcellular membranes.

An outline of the chemical structure of MTT and its coloured formazan following enzymic reduction is shown in **figure 3.viii.** overleaf. The reaction has been utilised for a number of *in vitro* bioassays. Most of these have not involved measurement of stimulated dehydrogenase activity *per se*, but have used MTT reduction as an indicator of cell populations in response to cytotoxic or mitogenic factors, Mosmann (1983).

### **c. Development of MTT assay for Hs68 fibroblasts**

#### **Cell culture conditions**

Growing stocks of adherant Hs68 cells were first removed from tissue culture flasks by the addition of the trypsin / EDTA solution. They were then seeded into 96-well microtitre plates in volumes of 150 µl/well complete medium at densities ranging from  $1 \times 10^4/\text{ml}$  ( $3 \times 10^3 / \text{cm}^2$ ) to  $5 \times 10^4/\text{ml}$  ( $1.5 \times 10^4/\text{cm}^2$ ), and incubated at  $37^\circ\text{C} / 5\% \text{CO}_2$  for 4 days. This incubation time was used to allow the cells to recover from passaging, adhere once again and begin their exponential growth phase.

The spent medium was subsequently removed by inverting and vigorously shaking the microtitre plate, and blotting the remainder onto sterile paper. Each well then received 100µl of serum-free DMEM + 0.1% BSA and the plate incubated for another 24 hours at  $37^\circ\text{C} / 5\% \text{CO}_2$ , principally to remove any remaining serum. Medium was again removed, and replaced by 100µl sample for a further 48 hours at  $37^\circ\text{C} / 5\% \text{CO}_2$ .

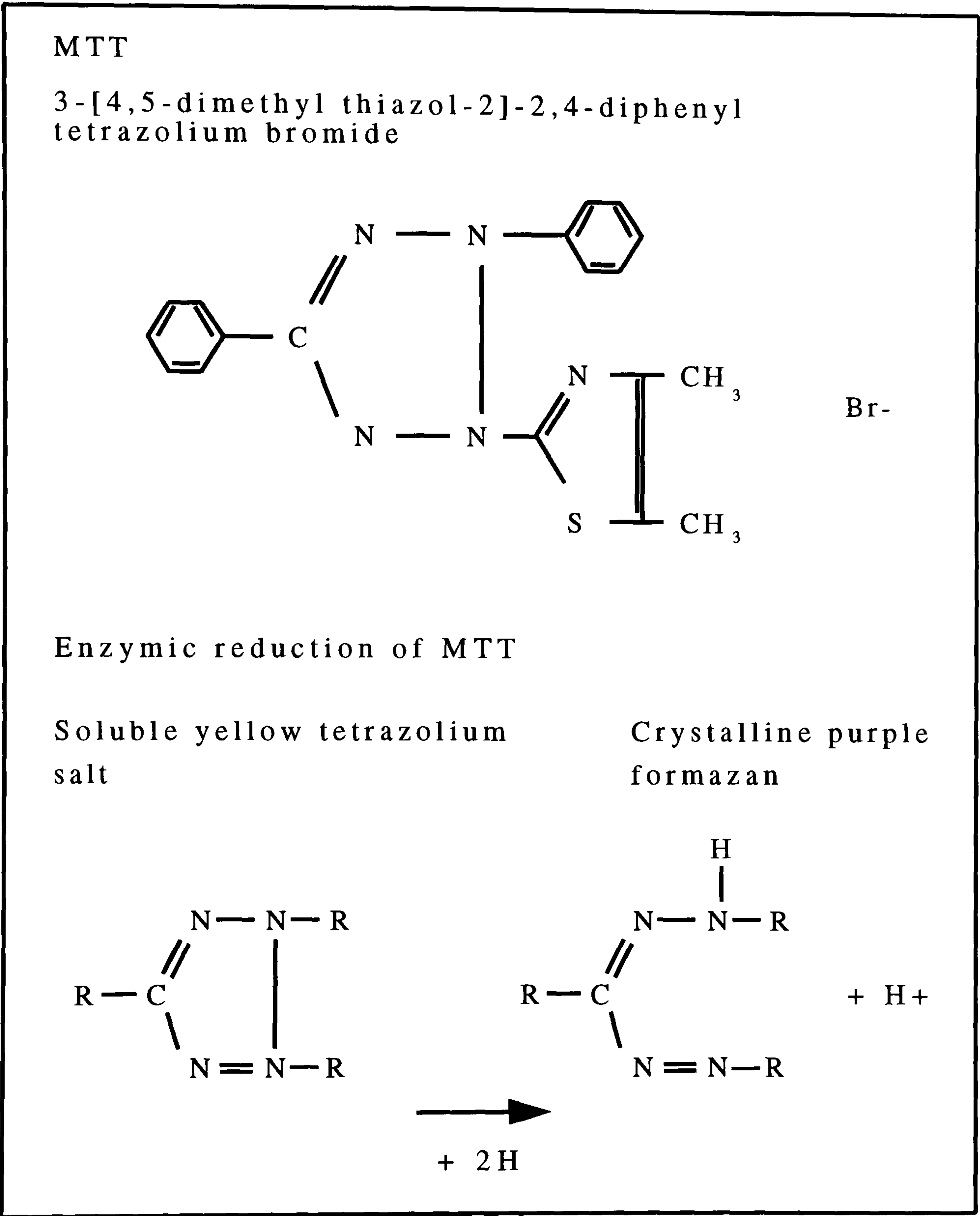
#### **MTT assay methodology**

MTT (5 g/l) was solubilised in Dulbecco's formula phosphate buffered saline (see page 70), containing both calcium and magnesium salts:

Calcium chloride	13 mg
Magnesium chloride	10 mg
H <sub>2</sub> O	100 mls



Figure 3.viii.



Structure of tetrazolium salt MTT and schematic of its reduction to a coloured formazan product.  
Adapted from Altman (1974).

Once fully solubilised, the MTT solution was warmed to 37°C and 10µl added to each well on top of the 100µl sample. Following incubation at 37°C in air, the crystalline formazan was eluted into the surrounding medium by the addition of an acidified detergent consisting of:

Triton X100	10% (vol/vol)
Hydrochloric acid (concentrated)	100 mmols/l
H <sub>2</sub> O	100 mls

Microtitre plates were then shaken for 10 minutes to mix each well, and finally the absorbances measured using a BioRad microtitre plate reader with a test wavelength of 595nm and reference wavelength of 650nm.

**Initial experiments**

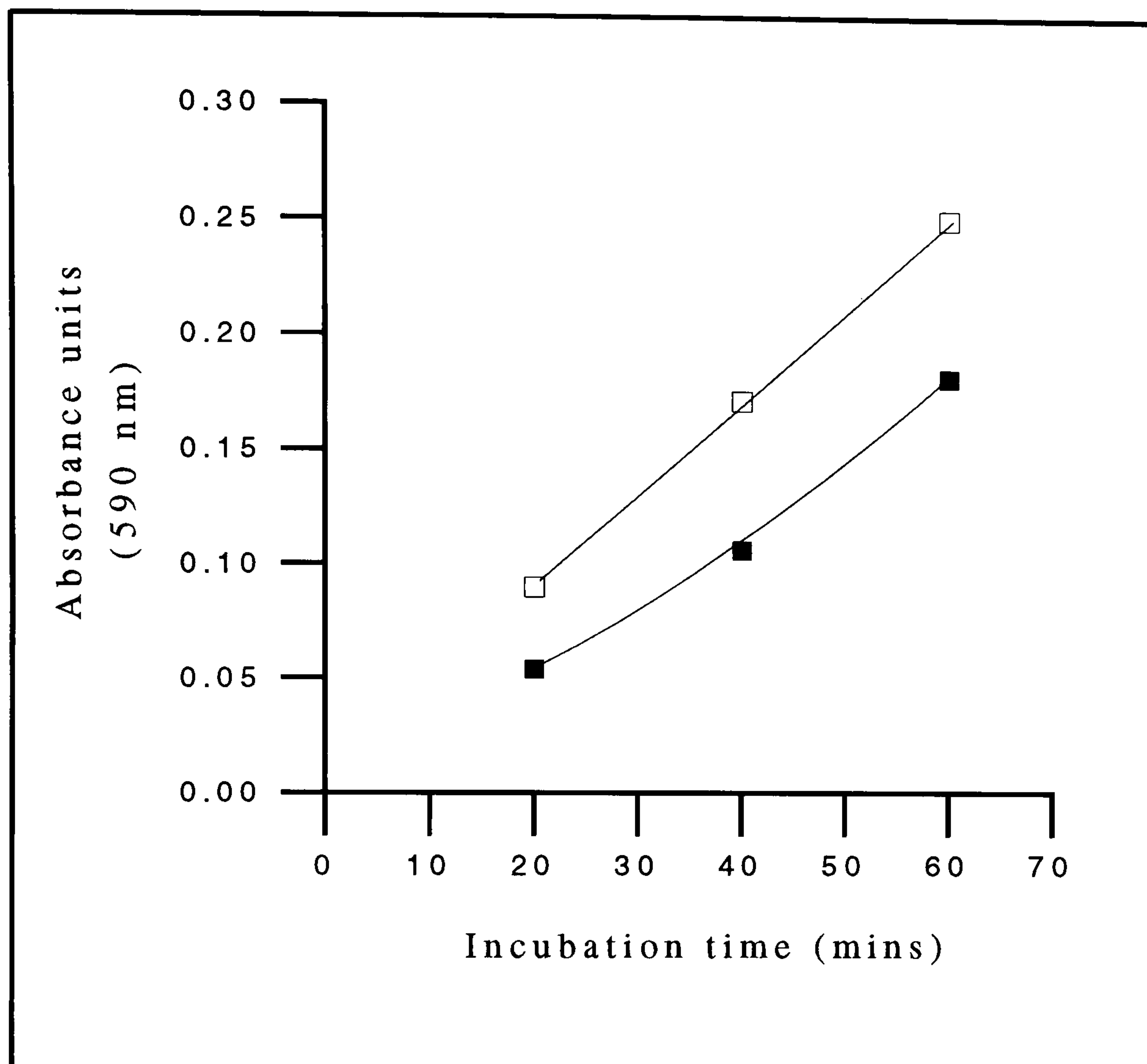
Time-course experiments using a final MTT concentration of 0.5 g/l, based upon Ealey *et al* (1988), and a higher dose of 1.0 g/l were first investigated. Cells were exposed to serum-free medium only for 48 hours before the addition of the MTT. As shown in **figure 3.ix.**, both the MTT concentrations resulted in fairly linear formazan production during the 20 to 60 minute incubations.

The relationship between cell number and MTT reduction was also investigated. As shown in **figure 3.x.**, formazan production was directly proportional to plating cell density, with very similar percentage increases seen after each incubation period.

For all subsequent assays, MTT was used at a concentration of 0.75 g/l over an incubation time of 30 minutes. Such conditions were chosen to give a reasonable level of formazan production (optical density of 0.1), and a convenient incubation period. Due to certain difficulties in solubilising MTT at the high dose of 1.0 g/l, the slightly lower dose of 0.75 g/l was chosen.

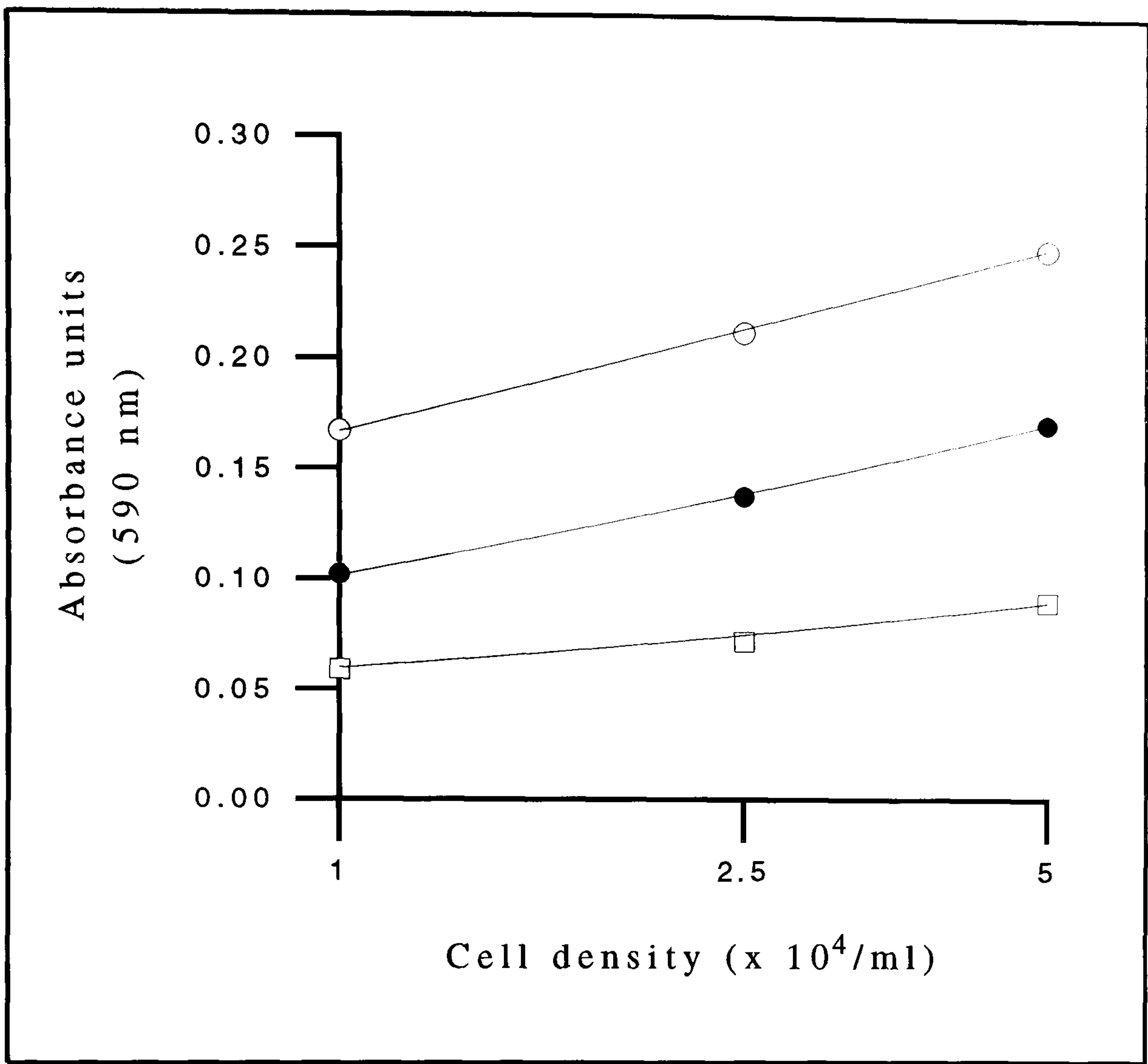


Figure 3.ix.



Time-course of Hs68 cell reduction of MTT tetrazolium salt to its coloured formazan. High density cells in serum-free media were exposed to either 0.5 g/l (■) or 1.0 g/l (□) MTT, and incubated for either 20, 40 or 60 minutes at 37°C. The reaction was stopped and coloured formazan eluted by the addition of acidified detergent Triton X100. Absorbances were spectrophotometrically read at a wavelength of 590nm. Mean results for duplicate wells are shown.

Figure 3.x.



The relationship between MTT formazan production and plating cell density of Hs68 cells following 48 hours in serum-free conditions. Results are shown as the mean absorbance at 590nm for duplicate cultures following MTT (1g/l) incubations of either 20 (□), 40 (●) or 60 (○) minutes at 37 °C.



### 3.9. STATISTICAL ANALYSIS

- i. Values from immunoassays and IGF bioassay were expressed as the means of triplicate tests unless otherwise stated. Error bars shown on graphs are standard deviation scores. The electrophoresis data shown was representative of duplicated experiments.
- ii. Radioimmunoassay data of fibroblast IGFBP-3 responses to cytokines were analysed by analysis of variance (ANOVA) using the S-PLUS computer package (Statistical Sciences Inc., Oxford, UK.) and Dunnett's multiple comparison test (Dunnett, 1955). This test is similar to a t-test but includes within it a constant term that depends on the residual degrees of freedom (given in the ANOVA) and the number of individual treatments being compared, including the control. An example is given below, using raw data on the effect of six doses of TGFβ on fibroblast IGFBP-3 taken from **figure 7.ii**.

#### ANOVA

	degree freedom	Sums of squares	Means of squares	Frequency	P
Groups	6	1604.49	267.414	47.7672	< 0.0001
Residuals	15	83.97	5.598		

To calculate Dunnett's constant ( $D_k$ ) then take:

residual degrees of freedom = 15

number of treatments = 7 (including control)

which gives a  $D_k$  value of 2.89 (taken from published tables) for a 2 sided test with 95% confidence interval.

The difference between the six TGFβ treatment means (mean IGFBP-3 radioimmunoassay values,  $x_{2-7}$ ) and the control mean ( $x_c$ ) is then considered significant at the 5% level if :

$$(x_2 - x_c) > D_k \sqrt{MSe (1/n_2 + 1/n_c)}$$

since all  $n_{2-7}$  values = 3 (triplicate cultures for each dose of TGFβ)

and  $n_c = 4$  we have :

$$(x_2 - x_c) > 2.89 \sqrt{5.598 (1/3 + 1/4)} = 5.222$$

making any treatment mean that differs from the control mean by more than 5.222 significantly different;

$x_2 - x_c = 16.0 - 15.8 = 0.2$	Not significant at dose 0.01 $\mu\text{g/l}$
$x_3 - x_c = 27.2 - 15.8 = 11.4$	Significant at dose 0.05 $\mu\text{g/l}$
$x_4 - x_c = 30.6 - 15.8 = 14.8$	Significant at dose 0.1 $\mu\text{g/l}$
$x_5 - x_c = 35.9 - 15.8 = 20.1$	Significant at dose 0.5 $\mu\text{g/l}$
$x_6 - x_c = 38.0 - 15.8 = 22.2$	Significant at dose 1.0 $\mu\text{g/l}$
$x_7 - x_c = 33.0 - 15.8 = 17.2$	Significant at dose 10.0 $\mu\text{g/l}$

We may conclude therefore that all TGF $\beta$  doses of 0.05  $\mu\text{g/l}$  and above significantly increase Hs68 fibroblast IGFBP-3 production compared to unstimulated controls.

iii. Analysis of variance (ANOVA) and Duncan's multiple range test (Duncan, 1955) were used to assess IGF-I bioactivity either in the presence or absence of cytokines in the MTT cytochemical assay. This test was chosen as it allows statistical analysis of both IGF-I doses compared to control and also all pairs of IGF-I doses between the cytokine treated and untreated cells. As this test controls the overall significance level, both of these two comparisons can be analysed all in one test. An example using raw data from **figure 8.viii.** showing the effect of 1  $\mu\text{g/l}$  TNF $\alpha$  on the MTT response to five doses of IGF-I is shown below.

ANOVA for MTT response to 5 doses of IGF-I, plus the untreated control, in the presence and absence of TNF $\alpha$ .

	df	SS	MS	F	P
Treatment	1	145.58	145.58	16.9	< 0.01
Dose	5	2258.45	451.69	52.5	< 0.01
Interaction	5	623.45	124.69	14.5	< 0.01



Residual	24	206.65	8.61		
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The ANOVA data therefore suggests strong evidence in terms of MTT response of a treatment effect (TNF $\alpha$  in this example), a dose effect (IGF-I) and an interaction between the two. More detailed analysis of each dose of IGF-I in the treated and untreated groups is then made using Duncan's multiple range test as follows;

First calculate standard error of each dose mean by

$$S_{y_i} = \sqrt{MS / n}$$

$$\text{in this example} = \sqrt{8.61 / 3} = 1.694$$

where Ms = residual mean square from ANOVA

and n = size of each dose group (triplicate cultures in this example)

Then obtain ranges **R**(p/f) for our data from Duncan's table of significant ranges using a 5% significance level (where p = number of means being compared (2,3,...12) and f = degrees of freedom of residuals (24)) and multiply each by  $S_{y_i}$ .

For this data set we calculate **R** as;

$$R_2(2,24) = 4.95$$

$$R_3(3,24) = 5.20$$

$$R_4(4,24) = 5.34$$

$$R_5(5,24) = 5.45$$

$$R_6(6,24) = 5.57$$

$$R_7(7,24) = 5.61$$

$$R_8(8,24) = 5.66$$

$$R_9(9,24) = 5.71$$

$$R_{10}(10,24) = 5.73$$

$$R_{11}(11,24) = 5.76$$

$$R_{12}(12,24) = 5.78$$

Comparisons are then made between the MTT data means obtained for the IGF-I dose points (designated  $Y_n$ ) in the presence or absence of TNF $\alpha$ . The notation was as follows;

	control	dose 5	dose 10	dose 25	dose 50	dose 100
IGF-I alone	$Y_1$	$Y_2$	$Y_3$	$Y_4$	$Y_5$	$Y_6$
IGF-I + TNF $\alpha$	$Y_7$	$Y_8$	$Y_9$	$Y_{10}$	$Y_{11}$	$Y_{12}$

These MTT means at each IGF-I dose are then sorted into decreasing order;

$Y_6$	$Y_{11}$	$Y_{12}$	$Y_{10}$	$Y_5$	$Y_9$	$Y_8$	$Y_4$	$Y_7$	$Y_1$	$Y_2$	$Y_3$
126.48	118.34	114.60	114.60	113.36	108.49	103.23	101.93	100	100	97.03	96.28

The largest difference is then compared to the largest Duncan range ( $R_j$ ) such that in this example;

$$\begin{aligned}
 Y_6 \text{ against } Y_1 &= 126.48 - 100 = 26.48 > R_9 = \text{significant at } p > 0.05 \\
 Y_5 \text{ against } Y_1 &= 113.36 - 100 = 13.36 > R_5 = \text{significant at } p > 0.05 \\
 Y_4 \text{ against } Y_1 &= 101.98 - 100 = 1.98 < R_2 = \text{not significant at } p > 0.05 \\
 Y_1 \text{ against } Y_2 &= 100 - 97.03 = 2.97 < R_2 = \text{not significant at } p > 0.05 \\
 Y_1 \text{ against } Y_3 &= 100 - 96.28 = 3.72 < R_3 = \text{not significant at } p > 0.05
 \end{aligned}$$

$$\begin{aligned}
 Y_{11} \text{ against } Y_7 &= 118.34 - 100 = 18.34 > R_8 = \text{significant at } p > 0.05 \\
 Y_{12} \text{ against } Y_7 &= 114.60 - 100 = 14.60 > R_7 = \text{significant at } p > 0.05 \\
 Y_{10} \text{ against } Y_7 &= 114.60 - 100 = 14.60 > R_7 = \text{significant at } p > 0.05 \\
 Y_9 \text{ against } Y_7 &= 108.49 - 100 = 8.49 > R_4 = \text{significant at } p > 0.05 \\
 Y_8 \text{ against } Y_7 &= 103.23 - 100 = 3.23 > R_4 = \text{not significant at } p > 0.05
 \end{aligned}$$

Therefore, in terms of relative change from control the MTT response significantly increased by dose 50  $\mu\text{g/l}$  IGF-I ( $Y_5$ ) in the absence of TNF $\alpha$ , and by dose 10  $\mu\text{g/l}$  IGF-I ( $Y_9$ ) in the presence of TNF $\alpha$ .

Comparisons can also be made between each IGF-I dose level in untreated or TNF $\alpha$



treated cells;

$$Y_8 \text{ against } Y_2 = 103.23 - 97.03 = 6.20 > R_5 = \text{significant at } p > 0.05$$

$$Y_9 \text{ against } Y_3 = 108.49 - 96.28 = 12.21 > R_7 = \text{significant at } p > 0.05$$

$$Y_{10} \text{ against } Y_4 = 114.60 - 101.98 = 12.62 > R_5 = \text{significant at } p > 0.05$$

$$Y_{11} \text{ against } Y_5 = 118.34 - 113.36 = 4.98 > R_4 = \text{not significant at } p > 0.05$$

$$Y_6 \text{ against } Y_{12} = 126.48 - 114.60 = 11.88 > R_3 = \text{significant at } p > 0.05$$

Therefore from these comparisons we can say that the relative changes in MTT response from control were significantly larger when IGF-I was in the presence of TNF $\alpha$  compared with being alone over the IGF-I dose range of 5, 10 and 25  $\mu$ g/l. At dose 50  $\mu$ g/l there was no significant difference in the relative change from control in the two groups, and at a dose of 100  $\mu$ g/l a higher relative change occurred in the absence of TNF $\alpha$ .

**CHAPTER 4. MECHANISMS OF IGF REGULATION**  
**OF IGFBP-3 *IN VITRO*.**



## **OVERVIEW**

With the probability that IGF-I stimulated increases in fibroblast IGFBP-3 are not due to alterations in *de novo* synthesis (as discussed in the Introduction), alternative mechanisms of regulation have been sought. It appears that a certain proportion of secreted IGFBPs, including IGFBP-1, IGFBP-2 and IGFBP-3, remain associated with the cell surface *in vitro* (McCusker *et al*, 1990), and that these may account for a large majority (up to 90%) of potential IGF binding sites (Clemmons *et al*, 1986). Recent data has now confirmed that IGFBP-1, via its RGD amino acid sequence, can bind to the integrin family of cell receptors, and also that IGFBP-3 and IGFBP-5 can be localised in the extracellular matrix (ECM) compartment (Jones *et al*, 1993), another possible storage site for secreted IGFBPs. Moreover, the relative abundance of basic amino acids in the carboxy-terminal region of IGFBP-3 suggests that it has the potential to bind with polyanionic sulfated glycosaminoglycans found in fibroblast ECM.

An attractive hypothesis to explain the non-IGF receptor mediated regulation of IGFBP-3 by IGF-I has now been proposed involving modulation of the cell-associated binding protein fraction (Martin *et al*, 1992). Following the observation that the IGF-I stimulated increase in IGFBP-3 in fibroblast conditioned medium was accompanied by a parallel reduction in cell-associated IGFBP-3, it was argued that IGF-I was simply displacing this store of binding protein. Clearly, no alteration in IGFBP-3 gene expression would be necessary in this mechanism, although no explanation was given to explain how such displacement occurs.

In the following chapter, an investigation has been made on the possible role that the recently discovered IGFBP proteases may have in mediating IGF-I regulation of IGFBP-3 secretion. Our motivation for these studies arose from certain reports over the last few years describing significant discrepancies between IGFBP-3 measured by radioimmunoassay and Western ligand blotting (Gargosky *et al*, 1992). It is believed that misleading low values in the latter technique result from the induction of proteolytic activity causing a reduction in the affinity of IGFBP-3 for the radiolabelled IGF ligand. Our initial studies, and those first published by Martin and Baxter (1991), seemed to suggest the opposite pattern existed for fibroblast IGFBP-3 in conditioned media following



IGF-I treatment. Our working hypothesis was therefore, that IGF-I was in some way inhibiting the activity of an IGFBP-3 protease produced by these cells leading to large exaggerated increases of binding protein in Western ligand blots, not dependent upon altered peptide secretion. We have therefore analysed the Hs68 CM samples for their IGFBP-3 protease activity and have investigated its possible down-regulation by IGF-I.

## **4.1. METHODS**

### **4.1.i. Cell culture**

Newborn human foreskin fibroblasts designated Hs68 were obtained from the American Type Tissue Collection, Rockville, USA. Cells were routinely cultured in Dulbecco's modified Eagles medium (DMEM) with 4.5 g/l glucose, 2 mmols/l L-glutamine and 10% fetal calf serum (FCS), in 5% CO<sub>2</sub> / 95% air.

### **4.1.ii. Conditioned medium (CM)**

CM was collected as follows : Hs68 cells were seeded into 6-well tissue culture plates (Nunc, Roskilde, Denmark) at a density of  $2 \times 10^4/\text{cm}^2$ . Cells were allowed to grow in DMEM + 10% FCS for 72 hours at 37°C. After this time cells were washed once in DMEM + 0.1% bovine serum albumin (BSA) and incubated in the same serum-free medium (SFM) for a further 24 hours. After another wash, 2mls SFM containing increasing doses of IGF-I was added to each well for 24 hours. CM samples were collected and immediately stored at -20°C until analysis of IGFBPs by WLB and IGFBP-3 RIA.

### **4.1.iii. Western ligand blotting (WLB)**

The effect of IGF-I on fibroblast IGFBP-3 protein secretion was assessed by WLB. In addition to native IGF-I similar experiments were also performed with the IGF-I analogue LongR3-IGF-I (GroPep Inc., Adelaide, Australia). LongR3-IGF-I is an 83 amino acid analogue of human IGF-I comprising the complete human IGF-I sequence with the substitution of an Arg for a Glu at position 3 and a 13 amino acid extension at the N-terminus. Whilst these modifications greatly reduce the affinity of the peptide for all IGFBPs they minimally affect IGF-I receptor binding. It therefore provides a useful tool



to delineate those effects mediated by the IGFs via cell membrane receptors or interaction with IGFBPs.

For all the WLB experiments 250µl pooled CM comprising SFM + IGF-I peptides was concentrated 5-fold before loading onto the gels. IGFBPs were visualised by the <sup>125</sup>I-IGF-I ligand as described in Methods.

#### **4.1.iv. IGFBP-3 radioimmunoassays (RIA)**

CM samples were also analysed by RIA using glycosylated human recombinant IGFBP-3 as tracer and standards, and three polyclonal IGFBP-3 antisera: SCH2/5, 1287-2-14 (Celltrix Inc., La Jolla, CA., USA), α-BP-3gl (provided by Dr.R.Rosenfeld, Stamford, CA., USA). No concentration of sample was necessary, with 50 µl CM assayed directly.

#### **4.1.v. IGFBP-3 gene expression**

Hs68 cells were plated in DMEM + 10% FCS into P100 culture dishes at a density of 10<sup>5</sup>cells/dish, 1.3 x 10<sup>3</sup>/cm<sup>2</sup>. After 96 hours at 37°C, cells were washed in SFM and incubated in same for 24 hours. After a further wash cells were incubated with test samples (5 mls/dish) for another 24 hours. Following removal of CM the cell monolayers were washed with sterile phosphate buffered saline (PBS) pH 7.4, before extraction of total RNA (RNAzol kit, Biogenesis, Bournemouth, U.K.) and subsequent Northern blotting for IGFBP-3 mRNA as described in the Methods chapter.

#### **4.1.vi. Analysis of protease activity**

IGF-I regulation of Hs68 protease activity capable of degrading radiolabelled <sup>125</sup>I IGFBP-3 was analysed by electrophoresis, following that of Lamson *et al* (1991), and as described in Materials and Methods. Cell culture and collection of serum-free CM was as described above using 24-well plates with the final 24 hour samples consisting of pooled CM from triplicate wells. The ability of IGF-I to alter the activity of the IGFBP-3 protease was assessed by adding either IGF-I or LongR3-IGF-I to the radiolabelled <sup>125</sup>I-IGFBP-3 substrate followed by incubations with 24 hour untreated CM for 3, 6, and 24 hours at 37°C.

Experiments were also performed to investigate whether the degradation of the IGFBP-3



substrate by Hs68 CM was artifactual, with fragmentation generated by the chemically disruptive SDS and heat (60-100°C) treatment used in both WLB and the electrophoresis technique. We therefore analysed  $^{125}\text{I}$ -IGFBP-3 following treatment with Hs68 cell protease by neutral fast-phase liquid chromatography (FPLC) utilising a 24 ml size-exclusion agarose column (Superose 12, Pharmacia, Uppsala, Sweden). Specifically, in a 1.5 ml siliconised centrifuge tube 200µl of Hs68 24 hour CM was incubated for 24 hours at 37°C with 150,000 cpm of freshly iodinated (< 7 days at -20°C) glycosylated  $^{125}\text{I}$ -IGFBP-3, in a total volume of 300µl. After this time FPLC was undertaken as described in Materials and Methods.

## **4.2. RESULTS**

### **4.2.i. Western ligand blot of Hs68 CM**

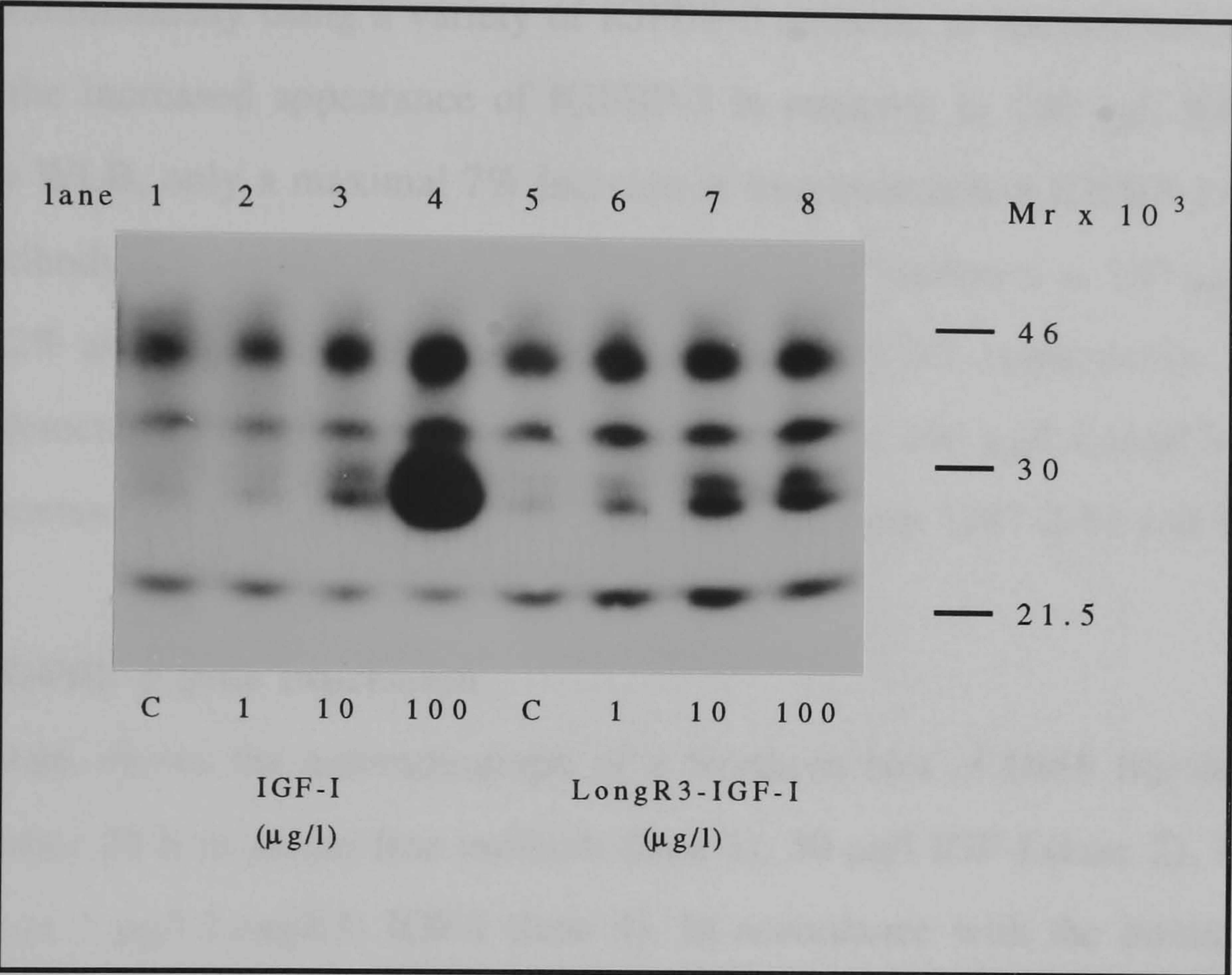
The effect of increasing doses of IGF-I upon IGFBPs in Hs68 CM is shown in lanes 1-4 in the autoradiograph in **Figure 4.i**. Under basal conditions of 24 hour SFM (lane 1) the fibroblasts secreted IGFBPs with apparent molecular weight of 43 kDa, consistent with IGFBP-3, a 33 kDa IGFBP, a 28-31 kDa consistent with IGFBP-5, and a 24 kDa IGFBP-4. Analysis of IGFBP mRNA species shown subsequently in this thesis confirmed the existence of IGFBP-3 and IGFBP-4, and similar but as yet unpublished experiments on these cells by Dr.Camacho-Hubner recorded IGFBP-5 mRNA and protein.

The addition of native IGF-I during the 24 hour incubation at doses of 10 µg/l (lane 3) and 100 µg/l (lane 4) affected the band intensity of all the aforementioned IGFBPs compared to the untreated control (lane 1). IGF-I at a dose of 100 µg/l caused an approximate 2-fold increase in IGFBP-3 abundance when the autoradiograph was assessed by scanning densitometry, and also had a similar although considerably greater effect upon the 28 kDa IGFBP-5. In contrast, IGFBP-4 was inhibited with increasing doses of IGF-I.

The effect of the IGF-I analogue LongR3-IGF-I is shown in lanes 5-8 in **Figure 4.i**. Basal 24 hour serum-free CM control from these experiments is shown in lane 5. LongR3-IGF-I up to a dose of 100 µg/l, was seen to have little or no effect upon the IGFBP-3 band intensity, or indeed IGFBP-4 or the 33 kDa band, despite being reported to be 10-20-fold



Figure 4.i.



Western ligand blot for IGFBPs in Hs68 cell conditioned medium following 24 hour treatment with either native IGF-I (lane 1-4) or LongR3-IGF-I (lane 5-8). Molecular weight markers are indicated.



more active in mitogenic bioassays. It had some stimulatory activity on the 28-30 kDa bands, although its effect was markedly smaller than that seen in response to native IGF-I.

#### **4.2.ii. IGFBP-3 radioimmunoassays**

The same Hs68 CM samples analysed by Western ligand blotting above were then subject to radioimmunoassay using a variety of IGFBP-3 antisera, as summarised in Figure 4.ii. Despite the increased appearance of IGFBP-3 in response to 100 µg/l IGF-I described above by WLB, only a maximal 7% increase in immunoreactive IGFBP-3 was observed using antibody α-BP-3gl. Moreover, maximal IGFBP-3 increases to 100 µg/l IGF-I were only 14.2% and 23.1% for antisera 1287-2-14 and SCH 2/5, respectively. Antiserum α-BP-3gl detected no increase in IGFBP-3 in response to 100 µg/l LongR3-IGF-I, whilst minor increases of 5.5% and 13.8% were seen by antisera 1287-2-14 and SCH 2/5.

#### **4.2.iii. IGFBP-3 gene expression**

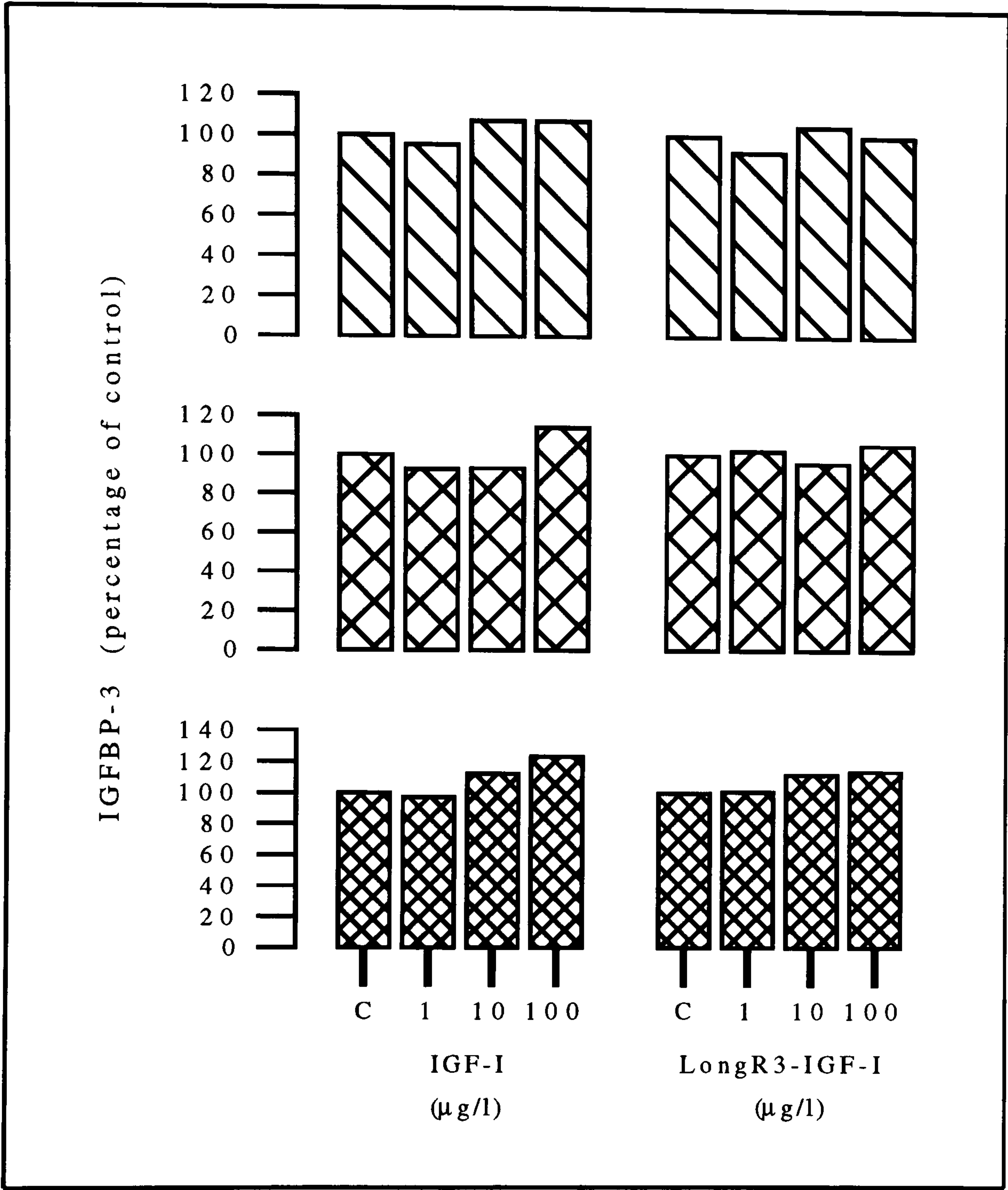
**Figure 4.iii.** shows the autoradiograph of a Northern blot of Hs68 fibroblast IGFBP-3 mRNA after 24 h in serum-free medium (lane 1), 50 µg/l IGF-I (lane 2), 1 mg/l insulin (lane 3) or 1 µg/l LongR3-IGF-I (lane 4). In accordance with the immunoassay data, native IGF-I did not cause any increase in the single 2.4-kb transcript recognised by the human IGFBP-3 cDNA probe, but actually lead to a moderate reduction. A similar effect was observed following treatment of the cells with the IGF-I analogue LongR3-IGF-I, but no alteration in IGFBP-3 gene expression was seen with insulin. As all of these peptides have the ability to interact with the type-I IGF receptors, it would seem that there is little or no regulation of IGFBP-3 at the gene transcription through this receptor. Also shown in **Figure 4.iii.** is the ethidium bromide staining of the 28s and 18s components of the 15 µg total RNA loaded in each lane, giving an approximation of the integrity of each sample and an indication of the accuracy of gel loading.

#### **4.2.iv. IGF-I regulation of IGFBP-3 protease(s)**

Proteolytic activity capable of leading to the breakdown of radiolabelled IGFBP-3, migrating at an approximate molecular weight of 35 kDa, was detected in 24 hour serum-free CM taken from Hs68 fibroblasts (**Figure 4.iv.**, lane 4). Whilst its activity was less



Figure 4.ii.




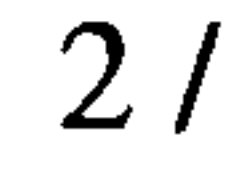
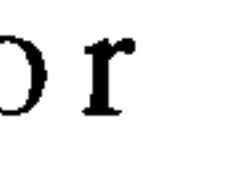
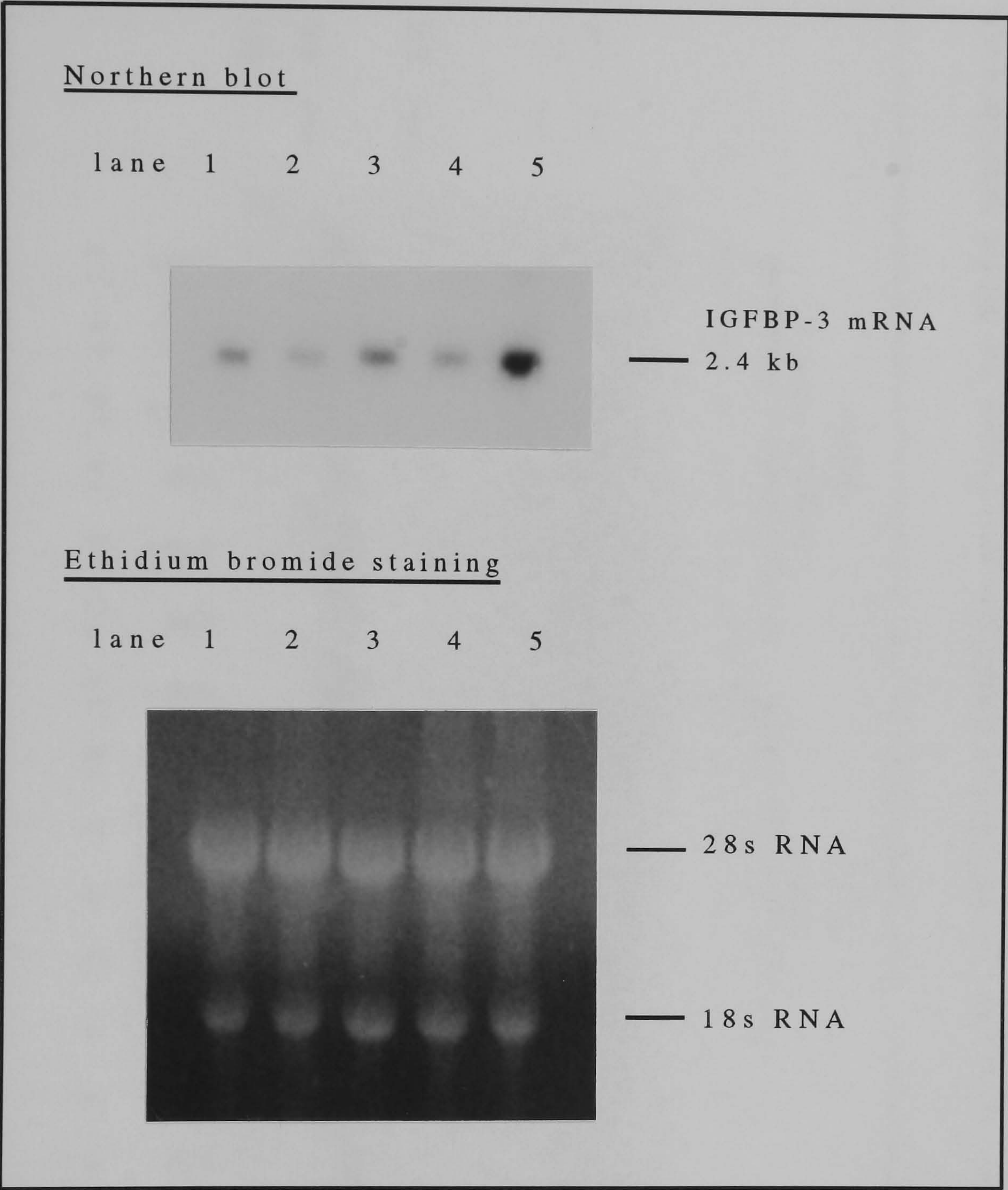
The effect of IGF-I upon immunoreactive levels of IGFBP-3 in Hs68 cell conditioned media, assessed by three different antisera. Cells were incubated for 24 hours in serum-free conditions with the addition of either native IGF-I or LongR3-IGF-I as described. Pooled samples from duplicate wells were then assayed by radioimmunoassay using antiserum  $\alpha$ -BP-3g1 () , antiserum 1287-2-14 () and antiserum SCH 2/5 () as described in the Methods. The mean error between duplicates was 5.1%.





Figure 4.iii.

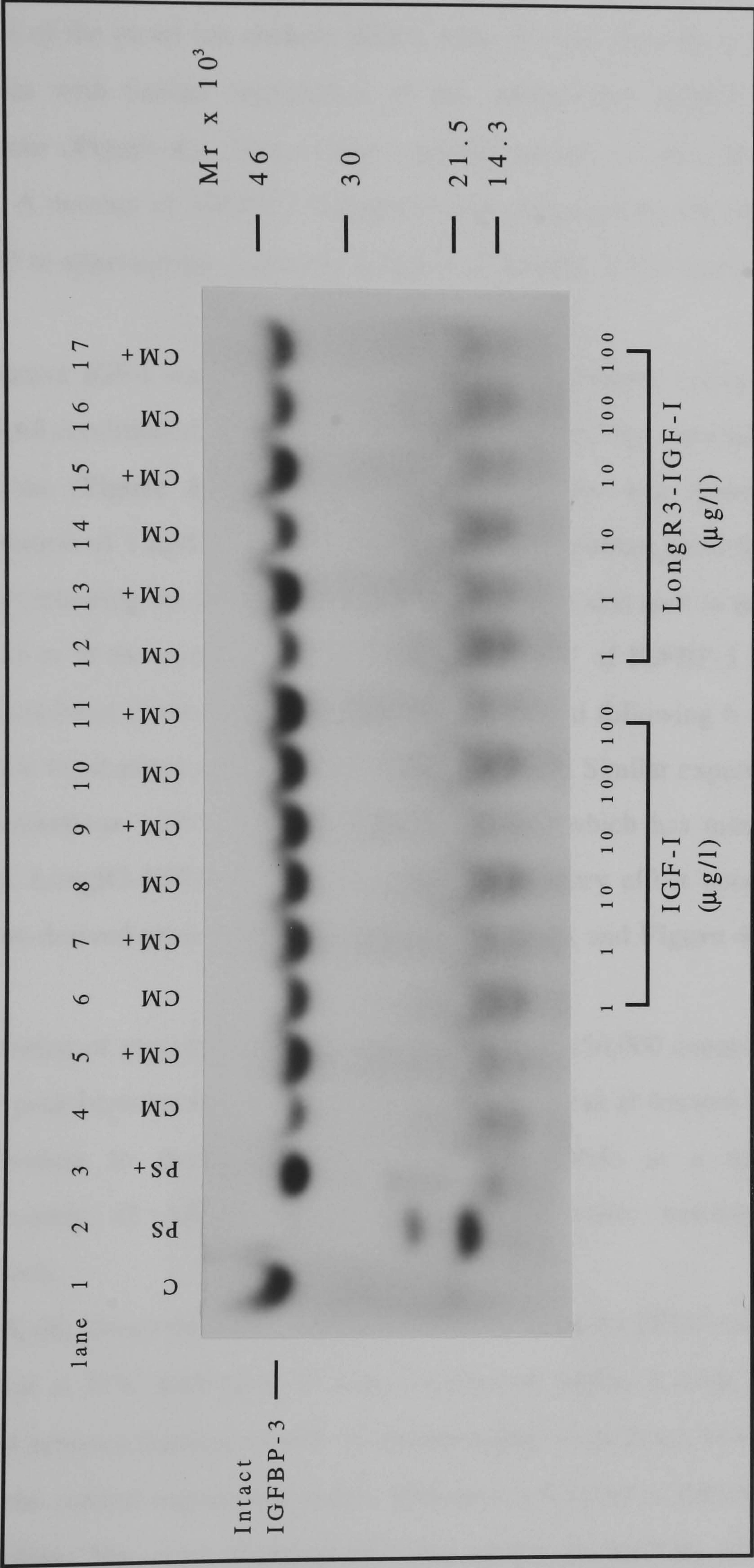


Northern blot showing IGFBP-3 mRNA abundance in Hs68 cells following 24 hour treatment with IGF-I and related peptides. Results are shown for control untreated cells (lane 1), 50µg/l native IGF-I (lane 2), 1mg/l insulin (lane 3), and 10µg/l LongR3-IGF-I (lane 4). Treatment with 1µg/l TGFβ 1 acted as a positive control (lane 5).

To confirm that similar amounts of total RNA were loaded ethidium bromide staining of the gel is shown.



Figure 4.iv.



The effect of IGF-I on the activity of IGFBP-3 protease produced by Hs68 fibroblasts. Twenty four hour conditioned media (CM) from untreated cells was collected and incubated for 3 hours at 37°C with <sup>125</sup>I-IGFBP-3 in the presence of increasing doses of IGF-I or LongR3-IGF-I, as indicated. Similar incubations were made in the presence of 20 mmols/l EDTA (shown by +). Term pregnancy serum (PS) acted as a positive IGFBP-3 protease control.



potent than the well recognised protease found in late pregnancy serum (lane 2), it too was evident following incubations of 3 hours at 37°C and could be abolished by the inclusion of the metal ion chelater EDTA (lane 5). The proteolytic effect was also time-dependent with further degradation of the radiolabelled IGFBP-3 following 6 hour incubations (**Figure 4.v.**, lane 4) and complete breakdown after 24 hours (**Figure 4.vi.**, lane 4). A number of IGFBP-3 fragments were generated by the fibroblast protease and migrated at approximate molecular weights of 20 kDa, 15 kDa and 10 kDa.

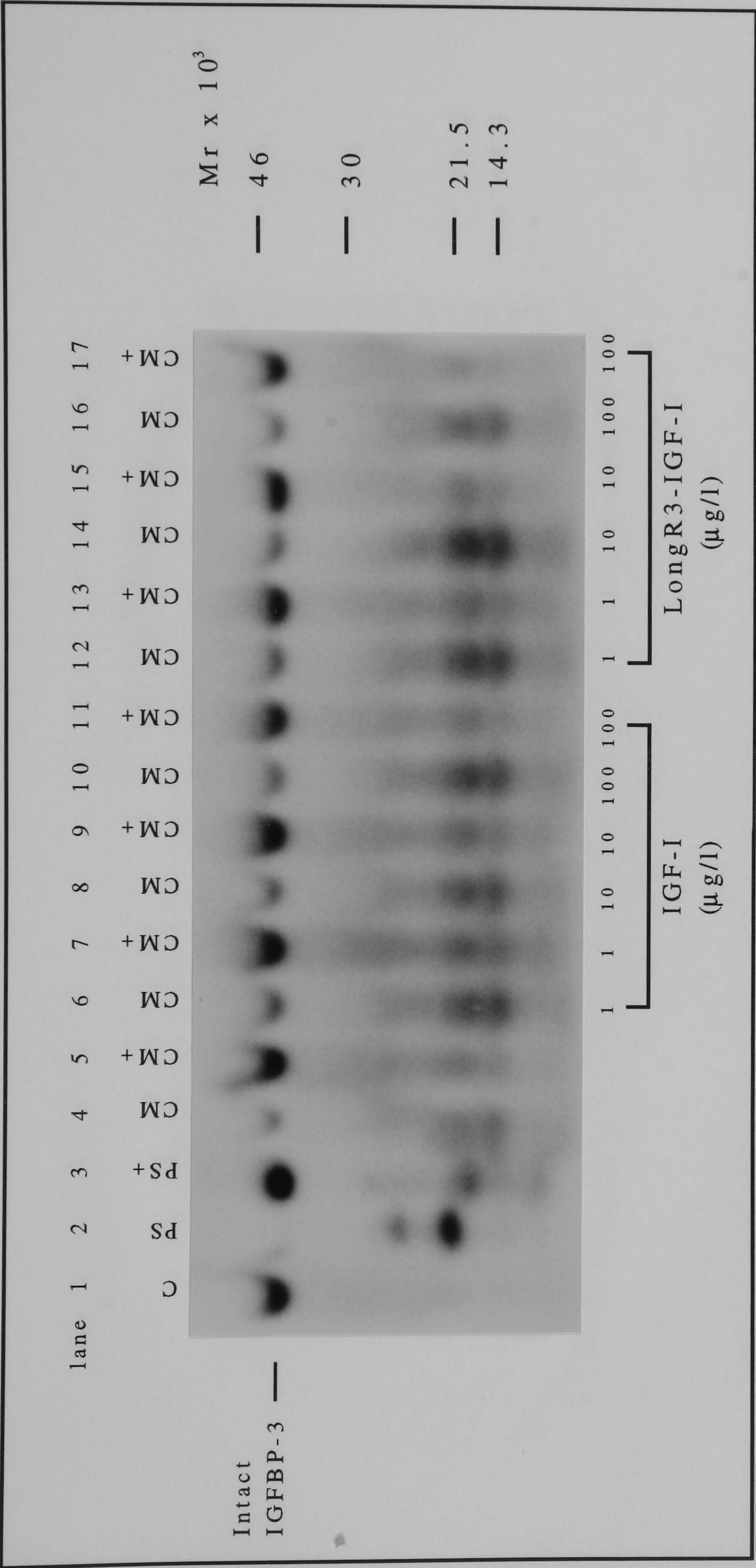
When native IGF-I was added to the  $^{125}\text{I}$ -IGFBP-3 substrate before the incubation with basal Hs68 conditioned media inhibition of protease activity was evident following 3 hour incubations (**Figure 4.iv.**). A dose-dependency effect was observed, with an IGF-I concentration of 1 µg/l producing visible protease inhibition (lane 6) and 100 µg/l IGF-I (lane 10) returning the level of intact IGFBP-3 back to that seen in the presence of EDTA (lane 11) or in the control (lane 1). This 'protection' of IGFBP-3 by native IGF-I was quite short-lived however, and no effect was observed following 6 and 24 hour IGFBP-3/protease incubations (**Figure 4.v.** and **Figure 4.vi.**). Similar experiments at 3, 6 and 24 hour incubations with the LongR3-IGF-I analogue which has minimal affinity for the IGFBPs, LongR3-IGF-I, failed to detect any inhibitory effect upon the activity of the fibroblast-derived protease (**Figure 4.iv.**, **Figure 4.v.**, and **Figure 4.vi.**).

Fractionation of glycosylated  $^{125}\text{I}$ -IGFBP-3 by FPLC (50,000 counts in 100 µl) produced a major peak between fractions 45-50 and a smaller peak at fraction 150+ (**Figure 4.vii.**), corresponding to the radiolabelled IGFBP-3 peptide at a molecular weight of approximately 45 kDa and the considerably smaller non-incorporated  $^{125}\text{I}$ iodide, respectively.

**Figure 4.viii.** shows the same  $^{125}\text{I}$ -IGFBP-3 run through the FPLC column following a 24h incubation at 37°C with Hs68 24 hour conditioned media. A large peak was once again observed between fraction 45 and 50 corresponding to the intact 45 kDa IGFBP-3 peptide seen in the control experiment above. However, a number of different sized peaks were also evident. The most abundant of these eluted in fractions 65 to 75 indicating a molecular weight of approximately 5 kDa. Other peaks were detected in fractions 30 to



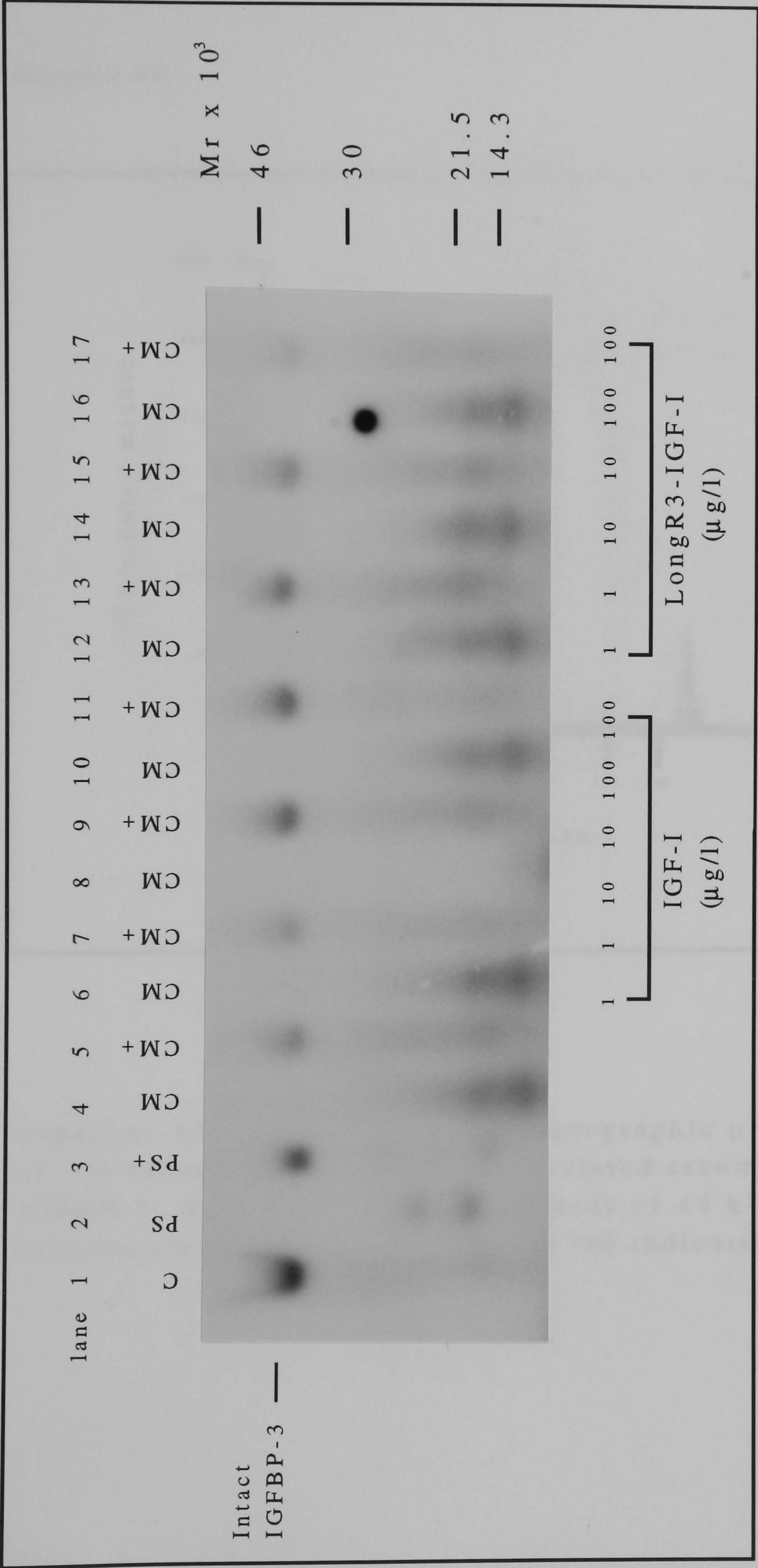
Figure 4.v.



The effect of IGF-I on the activity of IGFBP-3 protease produced by Hs68 fibroblasts. Twenty four hour conditioned media (CM) from untreated cells was collected and incubated for 6 hours at 37°C with <sup>125</sup>I-IGFBP-3 in the presence of increasing doses of IGF-I or LongR3-IGF-I, as indicated. Similar incubations were made in the presence of 20 mmols/l EDTA (shown by +). Term pregnancy serum (PS) acted as a positive IGFBP-3 protease control.



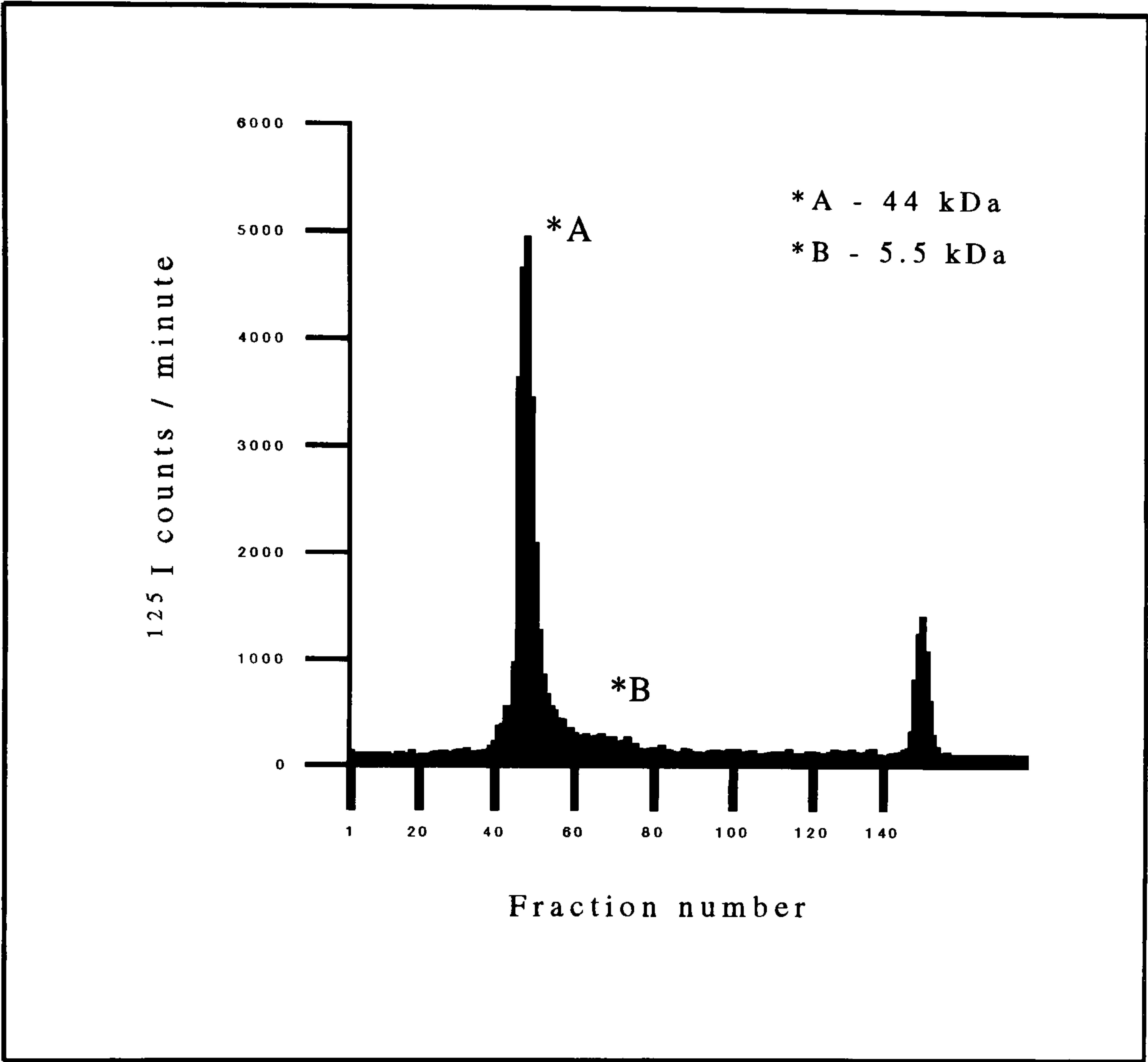
Figure 4.vi.



The effect of IGF-I on the activity of IGFBP-3 protease produced by Hs68 fibroblasts. Twenty four hour conditioned media (CM) from untreated cells was collected and incubated for 24 hours at 37°C with <sup>125</sup>I-IGFBP-3 in the presence of increasing doses of IGF-I or LongR3-IGF-I, as indicated. Similar incubations were made in the presence of 20 mmols/l EDTA (shown by +). Term pregnancy serum (PS) acted as a positive IGFBP-3 protease control.

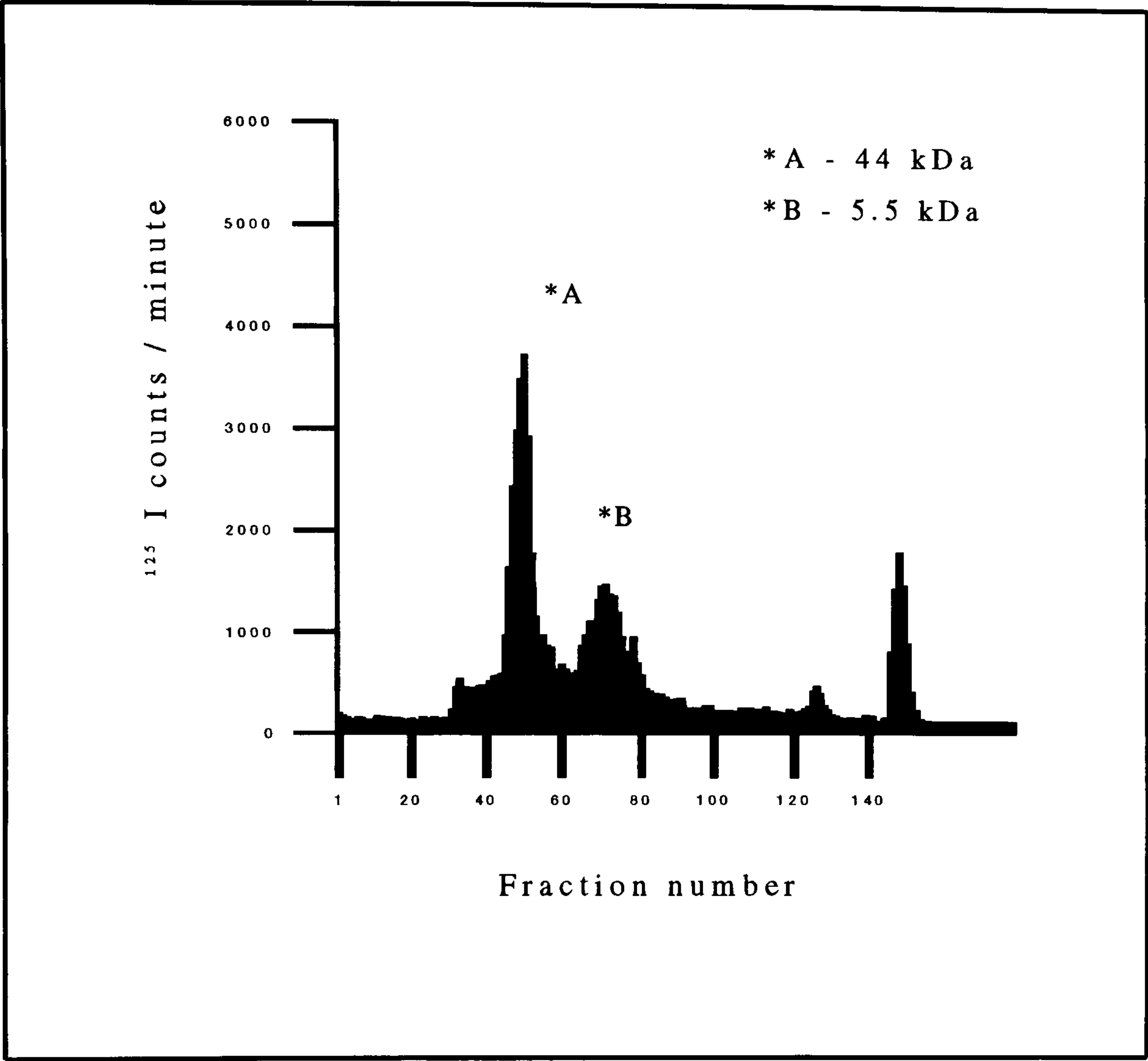


Figure 4.vii.



Superose-12 size exclusion chromatographic profile of  $^{125}\text{I}$ -radiolabelled human glycosylated recombinant IGFBP-3. Molecular weight standards of 44 kDa (ovalbumin) and 5.5 kDa (insulin) are indicated.

Figure 4.viii.



Superose-12 size exclusion chromatographic profile of  $^{125}\text{I}$ -IGFBP-3 following incubation for 24 hours at 37 °C with serum-free conditioned medium from untreated Hs68 cells. Molecular weight standards of 44 kDa (ovalbumin) and 5.5 kDa (insulin) are indicated



45, corresponding to one or more large molecular weight complexes in excess of 50 kDa. Inclusion of 20 mmols/l EDTA during the incubation lead to the reduction of the proportion of the major 5 kDa peak, confirming that this was a product of proteolytic cleavage (**Figure 4.ix.**).

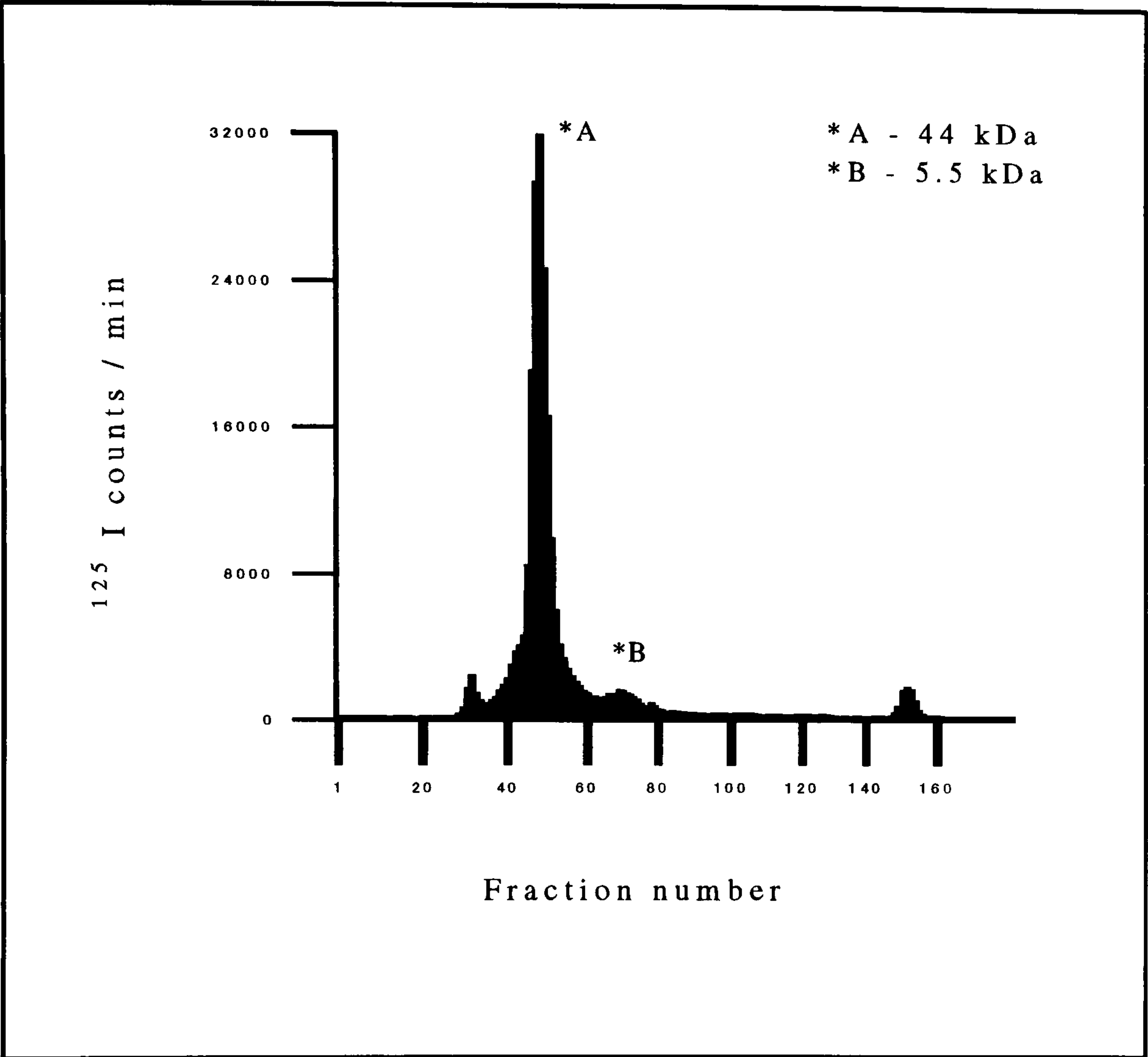
### **4.3. CONCLUSIONS**

The principal aim of these experiments was to clarify the post-translational mechanisms involved in IGF-I stimulation of IGFBP-3 secretion by human fibroblasts *in vitro*. With the recent discovery that a number of proteolytic enzymes are capable of degrading the IGFBPs, including IGFBP-3, we have attempted to examine if they play a part in this effect.

Firstly, we have established a normal human skin fibroblast model whose appearance of IGFBP-3 in conditioned medium responds to IGF-I in the expected manner. Apparent two-fold increases in IGFBP-3 were thus recorded in response to sub-maximal doses of IGF-I, when measured by Western ligand blot. As reported by others, this effect was not dependent upon IGF-I interacting with the type I IGF receptor, highlighted by the lack of effect of LongR3-IGF-I, a synthetic analogue with normal affinity for this receptor, but minimal affinity for the IGFBPs.

Further examination of these samples by radioimmunoassay using three specific IGFBP-3 antisera failed to detect comparable increases however. Although not directly reported elsewhere, such data is not totally without precedent. One of the first studies investigating IGF-stimulated enhancement of fibroblast IGFBP-3, from the only group using a radioimmunoassay (Baxter), reported minor increases of up to 50% only, despite much larger increases seen on Western ligand blot. Moreover, these increases were observed over an incubation period 3-fold greater than the one employed in our study; 72 hours compared to 24 hours. Whilst it is likely that real differences may exist between our neonatal Hs68 cell line and the neonatal fibroblasts used by this group, our experience with a number of IGFBP-3 antisera indicate that data showing such comparatively small increases in immunoreactive IGFBP-3 may also arise when the binding of said antibody is affected by IGF-I occupancy of the IGFBP-3. As described in the Methods section specific binding of one of the IGFBP-3 polyclonal antisera used in our study, 1287-2-14,

Figure 4.ix.



Superose 12 size exclusion chromatographic profile of  $^{125}\text{I}$ -IGFBP-3 following incubation for 24 hours at 37 °C with Hs68 conditioned medium in the presence of 20mmols/l EDTA. Molecular weight standards of 44 kDa (ovalbumin) and 5.5 kDa (insulin) are indicated.



was significantly reduced when in the presence of the IGF peptide. Presumably, this occurred because the epitope recognised by this antibody was very close to the IGF binding site on the IGFBP-3, causing a certain degree of competition. The net effect of high doses of IGF-I during the radioimmunoassay was therefore to reduce  $^{125}\text{I}$ -IGFBP-3 tracer binding, resulting in an artifactually increased sample IGFBP-3 value from the radioimmunoassay. Such interference could be overcome by adding a large excess of IGF to each assay tube. If the antibody used in the Baxter studies, designated R7, behaved in a similar manner this may perhaps explain the apparent dose-dependent increase in IGFBP-3 seen in response to IGF-I in their assay.

Furthermore, such an IGF-mediated reduction in binding affinity of IGFBP-3 for antibody could explain the reported concomittant 'displacement' of cell-associated IGFBP-3, proposed as the mechanism for IGF regulation of this binding protein. Simply, IGF-I may reduce the affinity of IGFBP-3 for antibody rather than its concentration. Notably in our study, the two antisera unaffected by the presence of IGF recorded no increase in immunoreactive IGFBP-3 in response to IGF-I.

Major discrepancies between IGFBPs measured by Western ligand blot and radioimmunoassay have recently been reported in pregnancy serum, cerebro-spinal fluid, seminal plasma, peritoneal fluid, amniotic fluid (Gargosky *et al*, 1992) and in the conditioned media from pheochromocytoma and anterior pituitary cells (Ocrant *et al*, 1992). They appear to describe the presence of one or more proteolytic enzymes capable of reducing the affinity of IGFBPs for the IGF molecule leading to low or even absent IGFBPs on Western ligand blots, despite normal immunoreactive levels of the peptides. Such discrepancies thus highlight the Western ligand blot technique as being a measure of both the molar concentration and binding affinity of the IGFBP.

Our data on the regulation of IGFBP-3 in Hs68 fibroblast conditioned medium suggested that the affinity of the binding protein, and not peptide concentration, was altered in the presence of IGF-I. However, despite recording the presence of IGFBP-3 proteolytic enzymes in the conditioned media taken from these cells, no alteration was seen following treatment with LongR3-IGF-I, suggesting that the 'stimulation' of IGFBP-3 by IGF-I seen on Western ligand blot was not due to down-regulated secretion of this protease. However,



we have subsequently shown that binding of native IGF-I to IGFBP-3, presumably leading to the formation of IGF-I/IGFBP-3 complexes, renders the binding protein partly resistant to degradation by the proteolytic activity produced by these cells. This was demonstrated by the addition of IGF-I to untreated Hs68 conditioned medium after it had been removed from the cells, which resulted in the inhibition of IGFBP-3 protease activity and may suggest that the protease was active at or near to the IGF binding site on the IGFBP-3 molecule. Such passive protection of the IGFBP-3 peptide may help explain the conflicting immunoassay and Western ligand blotting data presented in this chapter, but does not necessarily attach any important functional significance to IGF-I 'stimulation' of IGFBP-3 from these cells.

It does appear however from our neutral size exclusion gel chromatography data that proteolytic fragmentation of IGFBP-3 can occur under more physiological conditions than those necessary for electrophoresis. Experiments showed that incubation of glycosylated <sup>125</sup>I-IGFBP-3 with the Hs68 cell conditioned media lead to the appearance of a major fragment peak with an approximate molecular weight of 5 kDa, which could be mostly abolished with the addition of EDTA. Whilst this may suggest that proteolytic degradation of IGFBP-3 is possible under more physiological conditions (pH, absence of SDS and heat) we found no evidence of the major fragments of between 10 kDa to 20 kDa observed following electrophoretic separation. This may reflect differential migration of these fragments due to variation in glycosylation, although equally it may indicate that the large fragments are a product of the chemically harsh conditions and high temperature used in the SDS polyacrylamide gel electrophoresis and protease analyses.



**CHAPTER 5. FIBROBLAST IGFBP REGULATION BY**  
**TUMOUR NECROSIS FACTOR  $\alpha$**

## **OVERVIEW**

As outlined earlier the inflammatory cytokine TNF $\alpha$  is explicitly involved in a diverse range of effects in cell physiology. Moreover, many of these are involved in controlling cellular growth, with both positive and negative signals elicited. Evidence has accumulated to suggest some of these actions are mediated via the modulation of other growth factors found in serum, such as EGF and insulin (Vilcek *et al*, 1986). At present there is little data on the role this cytokine may have in regulating the IGFs, and it is this via investigation of its effect upon IGFBP secretion in the Hs68 normal human neonatal fibroblast model that has been addressed in the following chapter.

### **5.1. METHODS**

#### **5.1.i. Cell culture.**

The normal human neonatal skin fibroblast cell line, Hs68, was routinely cultured as described earlier.

#### **5.1.ii. Conditioned medium**

Cells were seeded into 24-well tissue culture plates in complete growth medium, (10% fetal calf serum), in a volume of 0.5 ml / well and at densities of 2.5, 5 x 10<sup>4</sup> / ml and 10<sup>5</sup> / ml. After 96 hours at 37°C in 5% CO<sub>2</sub>, the spent medium was aspirated and replaced by serum-free medium (SFM). SFM was essentially the same as growth medium, but 0.1% bovine serum albumin (BSA) replacing the fetal calf serum. Following a 24 hour incubation at 37°C, this medium was once again removed and the cells washed briefly with SFM. Test samples containing recombinant human TNF $\alpha$  in 300  $\mu$ l were then added to each well and incubated for a further 24 hours at 37°C. Triplicate wells were used for each sample. Conditioned medium was collected and immediately stored at -20°C until analysis.

#### **5.1.iii. Western ligand blotting**

IGFBPs in the above conditioned medium were analysed by the WLB technique as described in the Materials and Methods section, based on that by Hossenlopp *et al* (1986). Specifically for this set of experiments 600  $\mu$ l of pooled conditioned medium from



triplicate wells was lyophilised dried (4 hours in a GyroVap vacuum drier (Howe Ltd) and subsequently resuspended in 80 µl deionised water, thus giving a 6.66-fold concentration. A 30 µl volume of each sample was then added to 10 µl of 4-fold concentrated WLB sample buffer (see Methods page 73) to give a final concentration of the CM of 5-fold. After denaturation at 60°C, 25 µl was loaded into each lane on the polyacrylamide mini-gel and electrophoresed as previously described. After transfer of the proteins to nitrocellulose, the membrane was blocked for non-specific binding, incubated with <sup>125</sup>I-IGF-I, washed and dried. The blot was then subject to autoradiography for 3 days at -70°C.

#### **5.1.iv. IGFBP-3 radioimmunoassay**

The effect of TNFα on immunoreactive IGFBP-3 in Hs68 conditioned medium was quantified by specific radioimmunoassay (RIA). Human recombinant glycosylated IGFBP-3 (Celtrix, Santa Clara, CA) was used for standards and radiolabelling as described in Materials and Methods. For this set of experiments a rabbit polyclonal antiserum, 1287-2-14, was used at a final dilution of 1:20,000, with the additional presence of 2 ng/tube recombinant IGF-I for reasons described earlier. Due to the sensitivity of this assay no concentration of CM was necessary, with 50 µl measured directly.

#### **5.1.v. Analysis of IGFBP gene expression by Northern blotting**

Hs68 IGFBP-3 gene expression was analysed by Northern blotting, following the procedure described by Camacho-Hubner *et al* (1992). Hs68 cells were plated into 9.5 cm tissue culture petri-dishes in a volume of 10 mls and at a density of 10<sup>5</sup>/ml. After 96 hours at 37°C and 5% CO<sub>2</sub> the spent medium was aspirated and replaced with 5 mls SFM. Following 24 hours at 37°C this medium was aspirated and the cells washed briefly with SFM. TNFα samples in 5 mls SFM / dish were then added and incubated for either 1, 3, 6, 12 and 24 hours. After this time conditioned medium was collected and immediately frozen to -20°C for use in IGFBP-3 protease analysis as described below. RNA was isolated from the remaining cell monolayers and Northern blotting undertaken to determine IGFBP-3 and IGFBP-4 gene expression, as described in Materials and Methods.



#### **5.1.vi. Analysis of IGFBP-3 protease activity**

Conditioned medium from untreated and TNF $\alpha$  treated Hs68 cells as described above was incubated with  $^{125}$ I-IGFBP-3 for analysis of protease activity. Digestion incubations were kept at 37°C for 3 to 24 hours and subsequently electrophoresed, as described in the Methods chapter.

#### **5.1.vii. Cell surface-associated IGFBPs**

Initially  $^{125}$ I-IGF-I binding studies (as described in the Methods chapter) were performed on TNF $\alpha$  treated Hs68 fibroblasts to assess possible regulation of cell membrane IGF-I binding sites, potentially comprising type I, type II, insulin receptors and also cell-bound IGFBPs. The cell culture protocol was as described above, with cells treated with 1  $\mu$ g/l TNF $\alpha$  for 24 hours in serum-free conditions.

### **5.2. RESULTS**

#### **5.2.i. Western ligand blot**

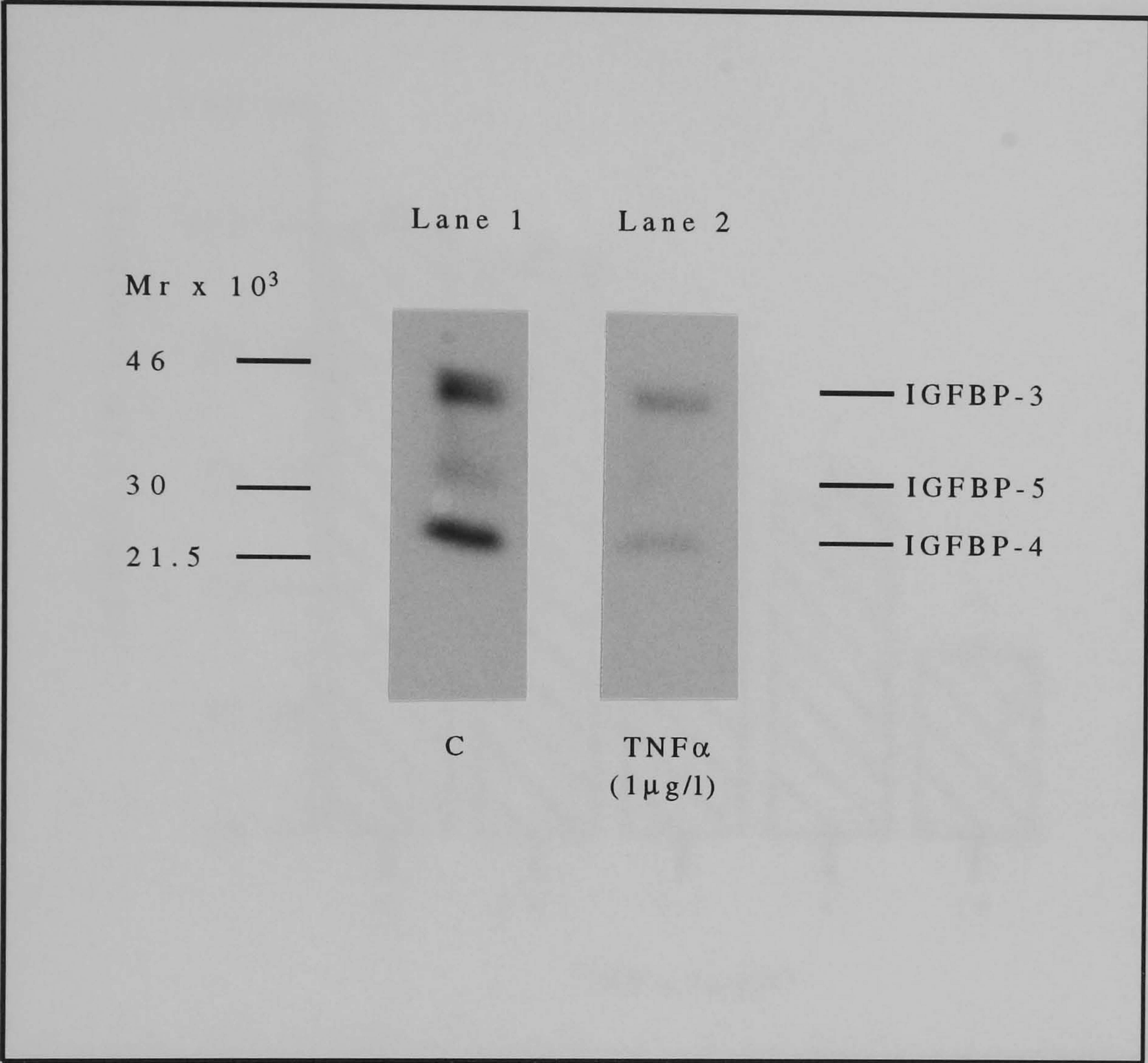
As shown in **figure 5.i.**, incubation of Hs68 fibroblasts for 24 hours in the presence of 1  $\mu$ g/l TNF $\alpha$  resulted in the reduction of conditioned media IGFBP band intensities on WLB. Scanning densitometric analysis of the autoradiograph revealed there to be decreases of 66.8% in the IGFBP-3 band (35 kDa), 81.1% in IGFBP-5 (28 kDa) and 81.7% in IGFBP-4 (24 kDa) compared with serum-free control values. Analysis of fibroblast cell number using both cell counting and our MTT cytochemical bioassay (described in Chapter 8. of this thesis) suggested that these effects were not simply due to TNF $\alpha$  induced cytotoxicity.

#### **5.2.ii. IGFBP-3 immunoreactivity**

Analysis of Hs68 24 hour conditioned medium by specific IGFBP-3 radioimmunoassay, shown in **figure 5.ii**, demonstrated that TNF $\alpha$  reduced the binding protein in a dose-dependent manner with significant effects seen at concentrations above 0.1  $\mu$ g/l. Variation in the absolute values of IGFBP-3 reduction were observed between experiments however, ranging from 25% to 60% inhibition at 1  $\mu$ g/l TNF $\alpha$ , although this did not appear to be correlated with cell passage number. Inhibition of IGFBP-3 by TNF $\alpha$  was not affected by



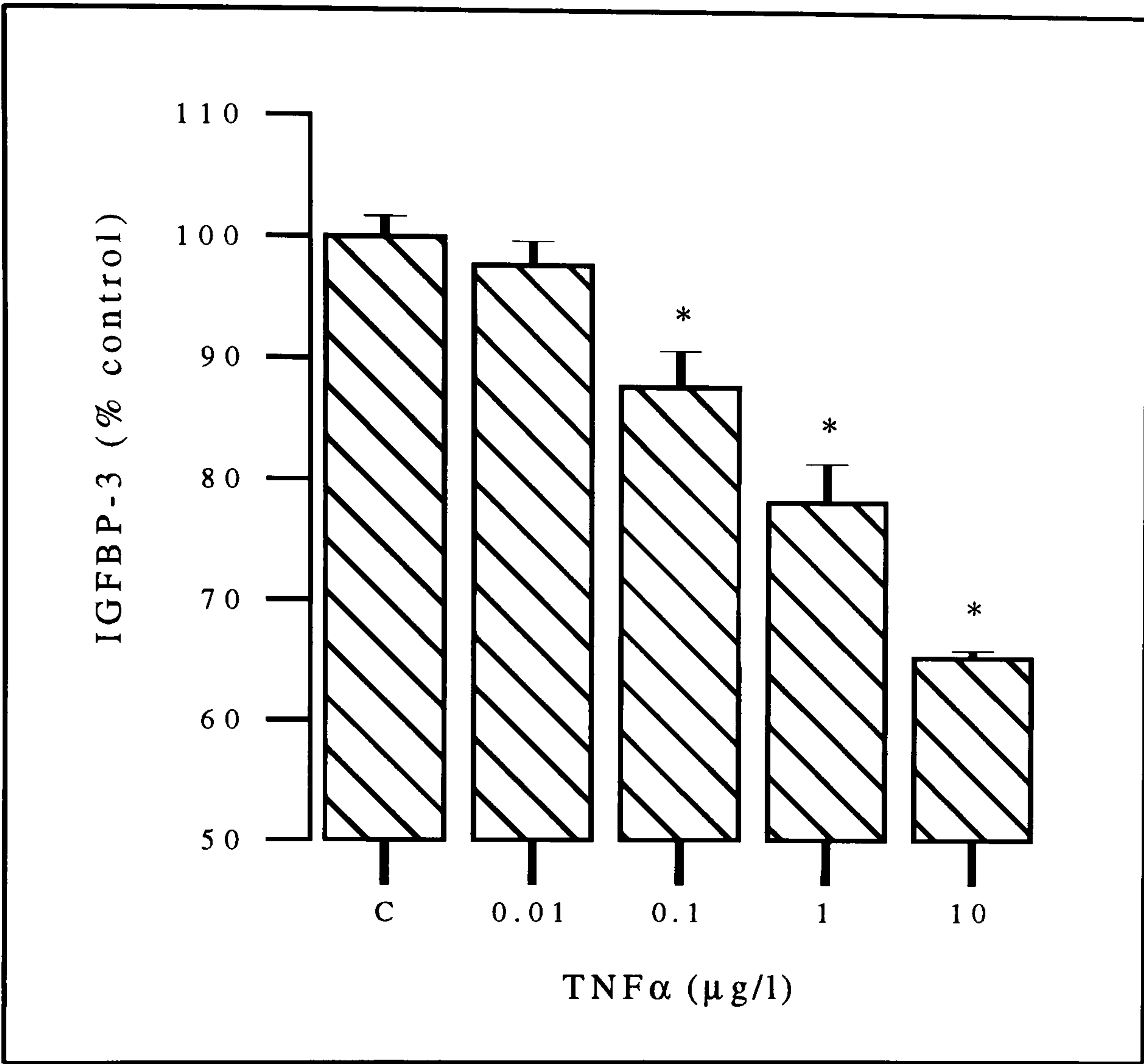
Figure 5.i.



Western ligand blot showing the effect of TNFα (1μg/l) on IGFBP secretion from Hs68 fibroblasts. Cells were treated for 24 hours in serum-free conditions. Lane 1 shows the untreated control, Lane 2 shows TNFα treatment. Molecular weight standards are indicated.



Figure 5.ii.



Dose-dependent TNFα-mediated inhibition of immunoreactive IGFBP-3 produced by Hs68 cells. Results are expressed as the percentage IGFBP-3, assessed by radioimmunoassay, of that found in 24 hour serum-free conditioned medium control  $\pm$  S.D. \*  $P < 0.05$  compared to control.



cell density, with similar responses of  $40.5\% \pm 15.8$ ,  $36.9\% \pm 6.5$ , and  $39.1\% \pm 6.4$  recorded in sparse ( $2.5 \times 10^4/\text{ml}$ ), semi-confluent ( $5 \times 10^4/\text{ml}$ ) and confluent ( $1 \times 10^5/\text{ml}$ ) cultures.

There are reports in the literature of the ability of  $\text{TNF}\alpha$  to stimulate the synthesis and secretion of prostaglandin  $\text{E}_2$  ( $\text{PGE}_2$ ) from fibroblasts, and certain physiological effects of  $\text{TNF}\alpha$  have now been attributed to the secondary effects of  $\text{PGE}_2$ . Although the inhibitory action of  $\text{TNF}\alpha$  on IGFBP-3 secretion was observed within 24 hours, perhaps suggesting it to be mediated directly by the cytokine, experiments were performed to investigate the possible role of  $\text{PGE}_2$  in this effect.

As shown in **figure 5.iii.**, following a 24 hour incubation with recombinant  $\text{PGE}_2$  immunoreactive Hs68 IGFBP-3 in conditioned medium was inhibited in a dose-dependent manner. Significant inhibition, similar to that caused by  $\text{TNF}\alpha$ , was observed at doses of 1 mg/l and above. However, the cyclo-oxygenase inhibitor indomethacin, which blocks the synthesis of the prostaglandins, had no effect on either the  $\text{TNF}\alpha$  effect or basal IGFBP-3 secretion over a dose range of 0.1 - 10  $\mu\text{M}$ . This would suggest that IGFBP-3 inhibition by  $\text{TNF}\alpha$  is not due to enhanced  $\text{PGE}_2$  synthesis.

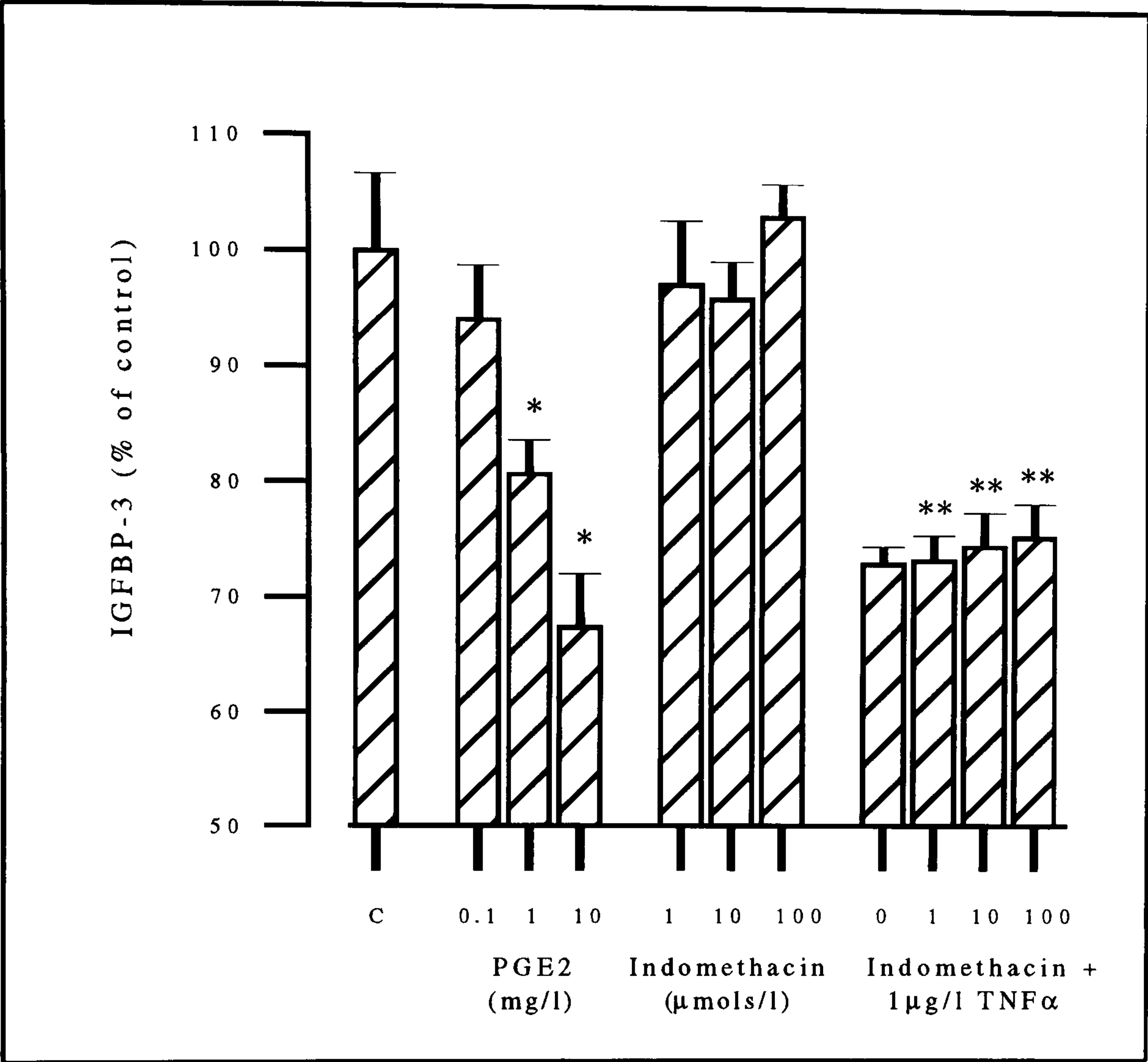
Interferon-gamma ( $\text{IFN-g}$ ) is a cytokine often closely associated with  $\text{TNF}\alpha$  *in vivo*. It was perhaps not surprising therefore in our *in vitro* model  $\text{IFN-g}$  also inhibited IGFBP-3 secretion from Hs68 fibroblasts, as seen in **figure 5.iv.** This effect was dose-dependent, with significant inhibitory activity observed at doses of 1  $\mu\text{g/l}$  or more, some ten-fold less potent than  $\text{TNF}\alpha$ . A number of *in vitro* studies have also shown that  $\text{IFN-g}$  can modulate the function of  $\text{TNF}\alpha$ , possibly via its ability to up-regulate cell membrane  $\text{TNF}\alpha$  receptors. The co-incubation of  $\text{IFN-g}$  with 1  $\mu\text{g/l}$   $\text{TNF}\alpha$  resulted in attenuated inhibition of Hs68 IGFBP-3 secretion, with 10  $\mu\text{g/l}$   $\text{IFN-g}$  fully reversing the  $\text{TNF}\alpha$  effect.

### **5.2.iii. IGFBP gene expression**

#### **IGFBP-3**

Despite the significant decrease in IGFBP-3 peptide levels in Hs68 conditioned media as measured above by both WLB and RIA, no parallel reduction in IGFBP-3 mRNA was

Figure 5.iii.



The inhibitory effect of prostaglandin E2 (PGE2) on IGFBP-3 secretion from Hs68 fibroblasts. The cyclo-oxygenase inhibitor indomethacin was without significant effect upon basal IGFBP-3 levels or upon the inhibitory effect of 1 μg/l TNFα.

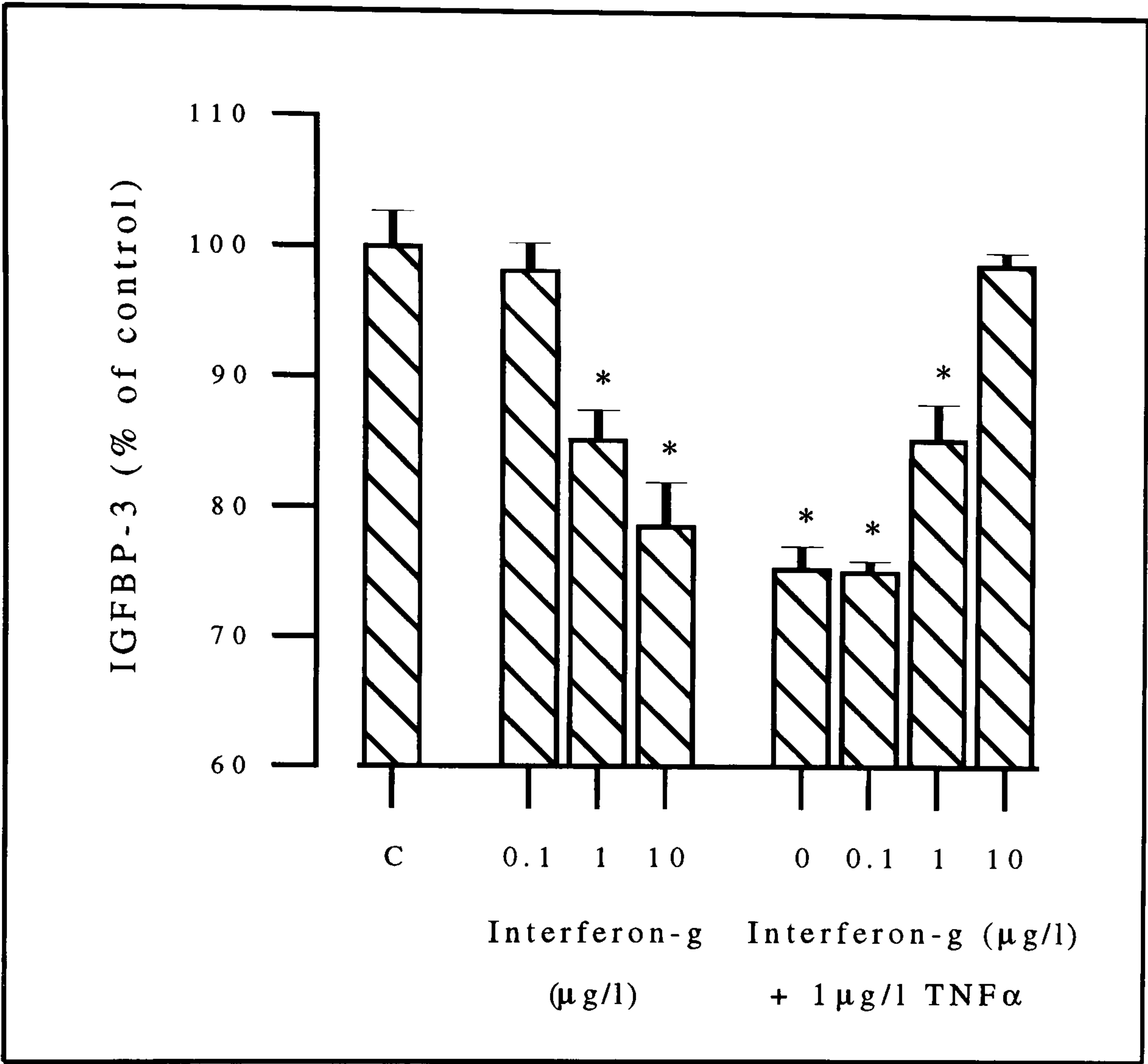
IGFBP-3 collected over 24 hours was assessed by radioimmunoassay, and results are expressed as the percentage of the serum-free control.

\* P < 0.05 compared to control

\*\* P > 0.05 compared to TNFα alone.



Figure 5.iv.



The effect of interferon-gamma on immunoreactive IGFBP-3 secretion from Hs68 fibroblasts, and its reversal of the inhibitory effect of TNFα. IGFBP-3 collected over 24 hours was assessed by radioimmunoassay, and results expressed as percentage of the serum-free control ± S.D.  
\* P<0.05 compared to control

observed following a 24 hour incubation with 1 µg/l TNFα (**figure 5.v.a.**). Indeed, scanning densitometric analysis revealed a small increase of approximately 1.5-fold in the 2.4 kb transcript identified by our <sup>32</sup>P-labelled IGFBP-3 cDNA probe. This effect could not be explained by variable gel loading of the total RNA (15 µg/lane) with minimal differences seen between the control and TNFα samples (**5.v.b.**).

Time-course studies also suggested there to be no consistent reduction in IGFBP-3 mRNA abundance in response to TNFα treatments of 2,4,6,8 or 12 hours, **figure 5.vi.** Due to problems of RNA loading in these particular experiments, B-actin mRNA levels are shown for comparison. These time-course studies were however also repeated on three separate occasions with cells at different passages, and with similar results. This would seem to confirm that TNFα mediated inhibition of Hs68 cell IGFBP-3 peptide was not a consequence of a parallel reduction in IGFBP-3 gene expression.

#### IGFBP-4

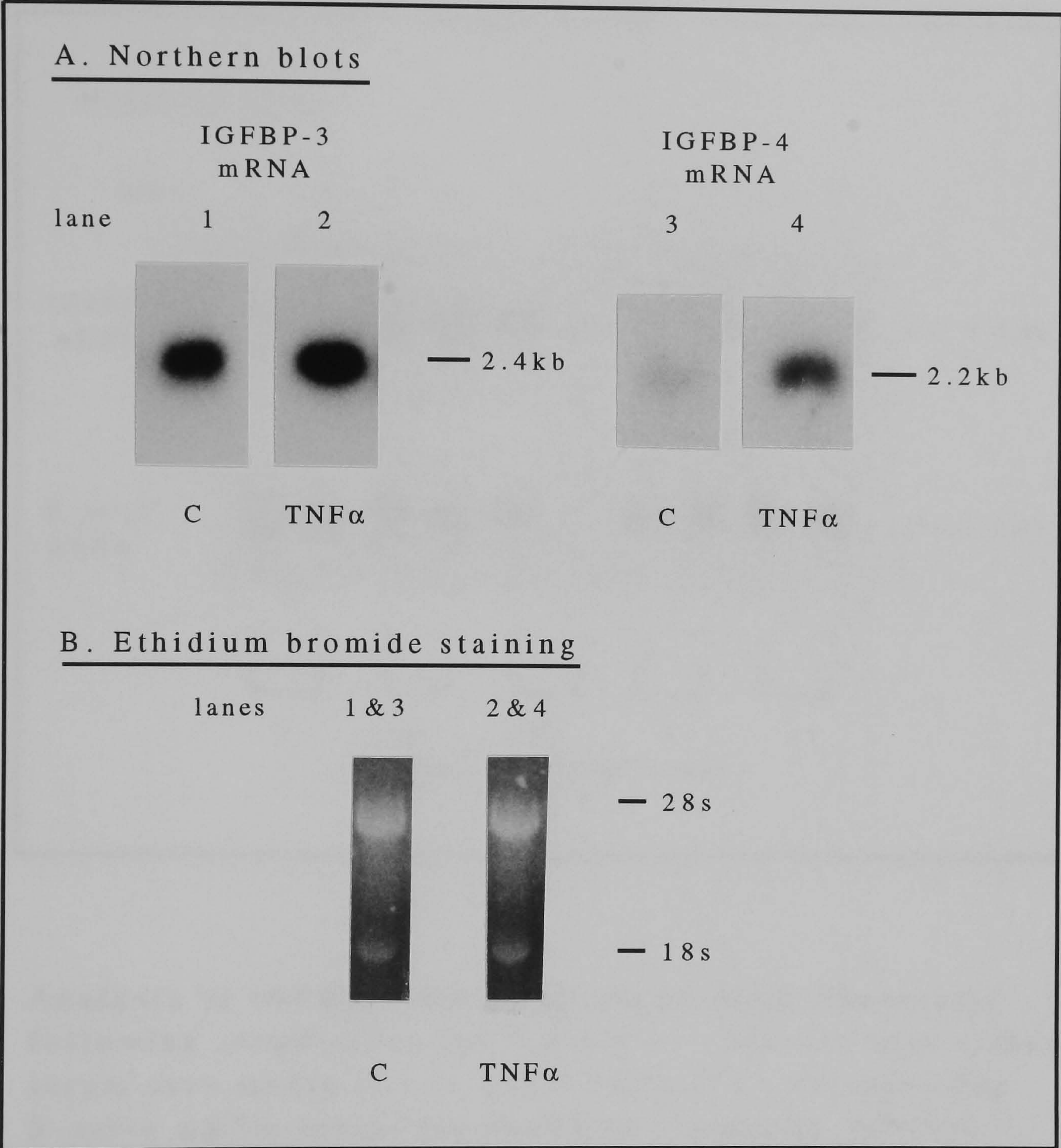
The membranes used in the analysis of IGFBP-3 gene expression regulation by TNFα above were re-hybridised with a <sup>32</sup>P-labelled IGFBP-4 cDNA probe. As shown in **figure 5.v.**, the intensity of the single 2.2 kb IGFBP-4 mRNA transcript was also increased following 24 hour treatment with 1 µg/ml TNFα.

#### 5.2.iv. IGFBP protease

The close agreement in data between WLB and RIA would suggest that the TNFα mediated reduction in IGFBP-3 involved real changes in peptide levels and was not via stimulation of proteolytic activity. However, as this cytokine is widely known to enhance the production of various fibroblast metalloproteinases, whilst concomitantly reducing associated inhibitors, experiments were performed to firmly establish the relevance of this function of TNFα on its ability to modulate IGFBP-3. As is illustrated in **Figure 5.vii.**, 24 hour treatment with 1 µg/l TNFα was seen to have no effect upon the activity of IGFBP-3 protease secreted by Hs68 cells, with no alteration in the degradation in <sup>125</sup>I-IGFBP-3 substrate compared to untreated cells.



Figure 5.v.



Regulation of IGFBP-3 and IGFBP-4 gene expression in Hs68 fibroblasts by 1 $\mu$ g/l TNF $\alpha$ . Shown in panel (A) are IGFBP-3 and -4 mRNA levels following 24 hours of treatment at 37°C. In panel (B) the ethidium bromide staining of the 28s and 18s RNA is shown to indicate the accuracy of loading 15 $\mu$ g total RNA per lane.



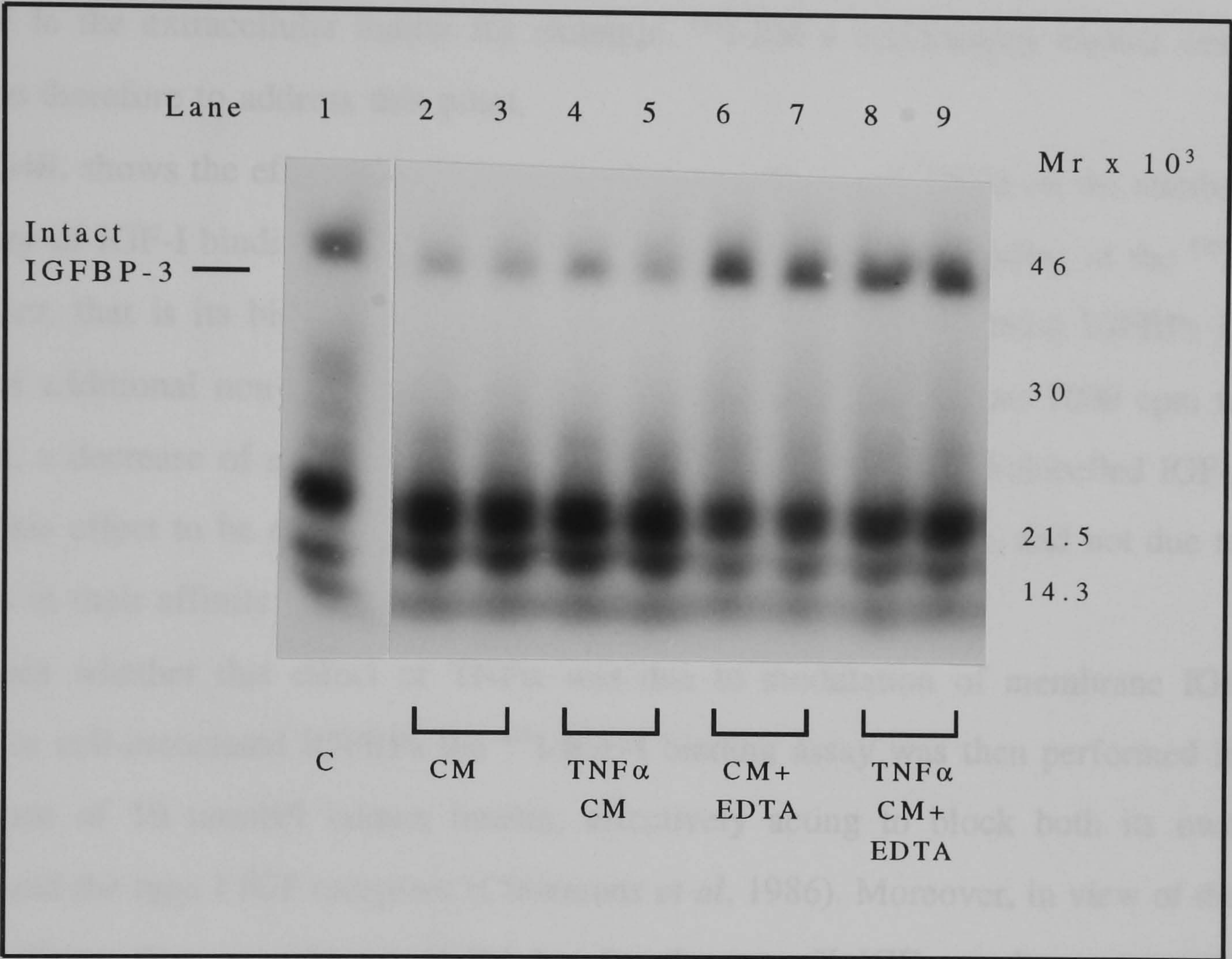
Figure 5.vi.



Analysis of IGFBP-3 mRNA levels in Hs68 fibroblasts following incubations for 2,4,6,8 or 12 hours with either serum-free media (C) or 1 $\mu$ g/l TNF $\alpha$  (T). Corresponding  $\beta$ -actin mRNA levels are shown to accurately indicate loading variations bewteen lanes.



Figure 5.vii.



The effect of TNF $\alpha$  on IGFBP-3 protease activity produced by Hs68 fibroblasts. 24 hour conditioned medium from untreated or TNF $\alpha$  (1 $\mu$ g/l) treated cells was incubated for 4 hours at 37 $^{\circ}$ C with <sup>125</sup>I-IGFBP-3 and subjected to electrophoresis. Lane 1 shows control IGFBP-3; lanes 2 and 3 show untreated CM; lanes 4 and 5 show TNF $\alpha$  treated CM; lanes 6 and 7 show untreated CM + EDTA (25 mM); lanes 8 and 9 show TNF $\alpha$  treated CM + EDTA.



### 5.2.v. Cell-associated IGFBPs

It is becoming increasingly apparent that in cell culture systems a potential mechanism for IGFBP regulation concerns the existence of cell-associated IGFBPs. A change in the levels of IGFBPs secreted into the surrounding medium, such as that elicited by TNF $\alpha$ , may arise through an alteration in the proportion of IGFBPs attached to the cell membrane or located in the extracellular matrix for example.  $^{125}\text{I}$ -IGF-I cell-binding studies were undertaken therefore to address this point.

**Figure 5.viii.** shows the effect of a 24 hour incubation with 1  $\mu\text{g/l}$  TNF $\alpha$  on the number and affinity of IGF-I binding sites in Hs68 fibroblasts. Specific total binding of the  $^{125}\text{I}$ -IGF-I tracer, that is its binding to membrane receptors and cell-associated IGFBPs in absence of additional non-radiolabelled IGF-I peptide, was reduced from 7000 cpm to 5370 cpm, a decrease of some 30%. Competitive binding with non-radiolabelled IGF-I revealed this effect to be due to a decrease in IGF binding site number, and not due to alterations in their affinity.

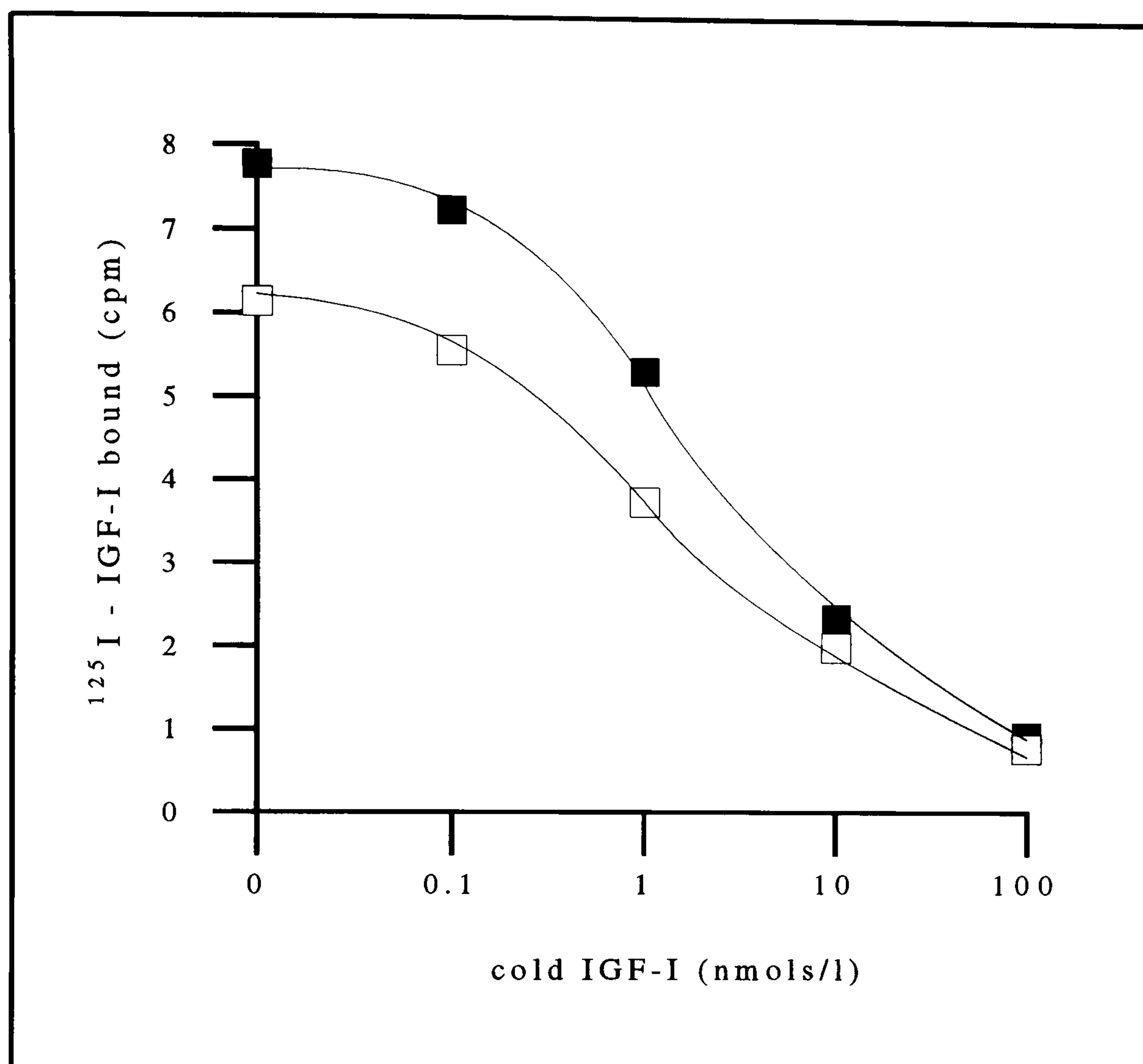
To ascertain whether this effect of TNF $\alpha$  was due to modulation of membrane IGF receptors or cell-associated IGFBPs the  $^{125}\text{I}$ -IGF-I binding assay was then performed in the presence of 10  $\mu\text{mol/l}$  human insulin, effectively acting to block both its own receptors and the type I IGF receptors (Clemmons *et al*, 1986). Moreover, in view of the minimal affinity that recombinant IGF-I has for the type II IGF membrane receptor (Rosenfeld *et al*, 1987.) it follows that the remaining available  $^{125}\text{I}$ -IGF binding sites must be the cell-associated IGFBPs. As described in **figure 5.ix.**, 24 hour TNF $\alpha$  treatment once again resulted in the reduction in  $^{125}\text{I}$ -IGF-I binding under the conditions just described. Specific binding of the tracer to IGFBPs was decreased some 30%, from the control 5260 cpm to 4350 cpm. Non-radiolabelled IGF-I displacement curves in both TNF $\alpha$  treated and untreated cultures were once again similar, suggesting a reduction in cell-associated IGFBP number, rather than affinity.

### 5.3. CONCLUSIONS

From the data described above it would seem that the inflammatory cytokine TNF $\alpha$  is capable of modulating IGFBP production by the Hs68 human skin fibroblast *in vitro*. Treatment with TNF $\alpha$  for 24 hours in serum-free conditions caused the apparent reduction

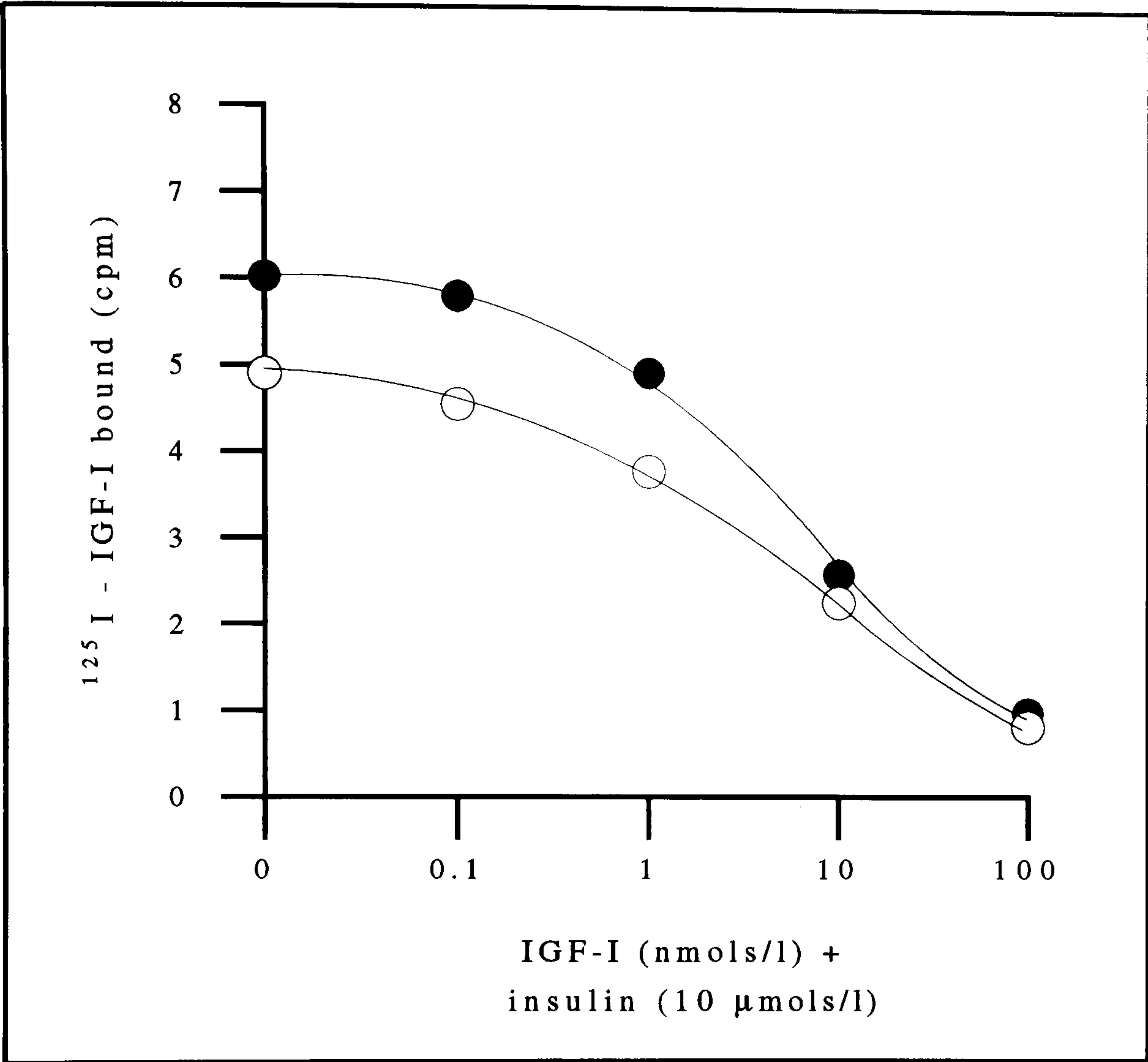


Figure 5.viii.



The effect of  $\text{TNF}\alpha$  on radiolabelled IGF-I binding to Hs68 fibroblast monolayers. Cells were incubated for 24 hours in the absence (■) or presence (□) of  $\text{TNF}\alpha$  ( $1\mu\text{g/l}$ ) and then IGF-I binding assessed as described in Methods.

Figure 5.ix.



The effect of  $\text{TNF}\alpha$  on radiolabelled IGF-I binding to Hs68 cell associated IGFBPs. Cells were incubated at  $37^\circ\text{C}$  for 24 hours in the absence (●) or presence (○) of  $\text{TNF}\alpha$  ( $1\mu\text{g/l}$ ) before binding of IGF-I was assessed with type-1 IGF and insulin receptors blocked with the addition of  $10\mu\text{mols/l}$  insulin.



of all IGFBPs secreted by these cells, IGFBP-3, IGFBP-4 and IGFBP-5, as determined by WLB. Confirmation that at least one of these alterations in IGFBP abundance involved changes in molar quantities of the peptide was provided by analysis of IGFBP-3 in conditioned media by specific radioimmunoassay (RIA) unaffected by proteolytic activity. In a similar fashion to the WLB data, IGFBP-3 was seen in the RIA to be typically reduced by approximately 35% following TNF $\alpha$  treatment.

This effect may not however be a direct one as TNF $\alpha$  can stimulate the synthesis of prostaglandins from a number of cultured cells, including adipocytes (Hardarddottir *et al*, 1992) and fibroblasts (Hori *et al*, 1991). Exogenous PGE<sub>2</sub> in our Hs68 fibroblasts was seen to inhibit IGFBP-3 secretion, suggesting therefore that the TNF $\alpha$  effect may indeed act via this pathway. However, addition of the cyclo-oxygenase inhibitor indomethacin, at doses at and above those found effective in blocking prostaglandin synthesis by others (Goss *et al*, 1993), was without effect on either basal or TNF $\alpha$  inhibited IGFBP-3 secretion, suggesting this not to be the case. It may be that either TNF $\alpha$  did not affect prostaglandin synthesis in Hs68 fibroblasts (data unavailable) or that after 24 hours in the presence of the cytokine insufficient PGE has been secreted to match the high doses of prostaglandin E<sub>2</sub> (>1 mg/l) found effective at inhibiting IGFBP-3 when added exogenously. Data supporting the latter explanation has been reported for TNF $\alpha$  mediation of lipolysis in cultured 3T3-F442A fat cells (Hardarddottir *et al*, 1992). The importance of TNF $\alpha$ -stimulated PGE<sub>2</sub> synthesis in contributing to the inhibitory effect on IGFBP-3 may increase over longer time periods than we have examined however.

Interferons have a number of physiological functions, in addition to their inhibitory action on virus replication, and these often involve the alteration in cellular growth. Interferon-gamma (IFN-g) has the capacity to modulate the activity of TNF $\alpha$ , enhancing its cytotoxicity in HeLa carcinoma cells (Stone-Wolff *et al*, 1984) and conversely inhibiting its mitogenic action in FS-4 normal foreskin fibroblasts (Vilcek *et al*, 1986). Taken together with the observation that IFN-g could completely block the TNF $\alpha$ -mediated reduction in IGFBP-3 secretion from Hs68 cells an attractive hypothesis would be that IFN-g acts as a negative growth signal. However, this is based on the assumption that IGFBP-3 is inhibitory to the mitogenic effects of IGF and also fails to account for the



inhibition of IGFBP-3 elicited by IFN- $\gamma$  when incubated alone.

Analysis of IGFBP-3 and IGFBP-4 gene expression in these cells following incubation with TNF $\alpha$  demonstrated that this effect was more complex than straightforward alteration in transcription of the respective mRNAs. Both the single transcripts for IGFBP-3 (2.4 kb) and IGFBP-4 (2.2 kb) were *increased* at the end of the 24 hour TNF $\alpha$  treatment, and no consistent down regulation of mRNAs was seen at earlier time-points in the incubation period. This apparent novel regulation of the IGFBPs therefore lead us to examine possible TNF $\alpha$  regulation of post-transcriptional mechanisms.

Data from others has shown that cultured fibroblast monolayers secrete one or more binding proteins that alter the cellular binding of IGF-I (Clemmons *et al*, 1986) and Jones *et al* (1993) has revealed that certain IGFBPs, including IGFBP-5 and IGFBP-3, can be found closely located with the fibroblast cell membrane or in the extracellular matrix. As described in the previous chapter, regulation of this immobilised store of IGFBPs has now been given as a mechanism to explain the increase in IGFBP-3 in fibroblast conditioned medium following IGF-I treatment (Martin and Baxter, 1992). One possible mechanism employed by TNF $\alpha$  may be via changes in the compartmentalisation of the IGFBPs therefore, that is the alteration in the proportion of cell-associated IGFBPs in comparison with that found in the conditioned medium. Rather than TNF $\alpha$  increasing the amount of cell-bound IGFBPs however, which would have perhaps explained our WLB and RIA data, a decrease was observed suggesting once again therefore reduced IGFBP secretion. An increase in IGFBP clearance by the fibroblasts is a possible alternative explanation but at this moment in time is purely speculative.

Even though the IGFBP-3 RIA used in these experiments was unaffected by the presence of IGFBP proteolytic activity produced by untreated Hs68 cells (see Methods section) the possibility existed that TNF $\alpha$  was stimulating separate distinct enzymic activity that affected our interpretation of the RIA data. Evidence that this cytokine can indeed upregulate a number of metalloproteinases, as outlined in the Introduction, gave a certain degree of support to this hypothesis. Analysis was therefore made of such proteolytic activity in Hs68 conditioned media following TNF $\alpha$  treatment using  $^{125}\text{I}$ -IGFBP-3 as substrate. Despite time-courses and various assay incubation periods no alteration in



IGFBP-3 degradation was observed however, suggesting that TNF $\alpha$  regulation of IGFBP-3 was not through mediation of a protease or associated inhibitor. In view of the well established role that TNF $\alpha$  has in mediating metalloproteinase activity, particularly in fibroblasts, such data was disappointing as regards ascribing a physiological role for the IGFBP-3 protease produced by our cells.

In conclusion, it would seem from the above data that perhaps the most likely mechanism to explain the apparent post-transcriptional regulation of IGFBP-3, and possibly IGFBP-4 by TNF $\alpha$  involves an effect upon the translation of the IGFBPs mRNA transcripts. Although not studied in the course of this thesis it follows that a decrease or inhibited rate of translation could result in a corresponding reduced rate of peptide secretion as we have observed. Moreover, it may also help explain the increases in IGFBP mRNA abundance seen following exposure to the cytokine, as this mechanism would perhaps cause an accumulation of untranslated mRNAs. Further studies will be necessary to address such a hypothesis and establish the precise mechanism of TNF $\alpha$  mediated down-regulation of fibroblast IGFBP secretion.

**CHAPTER 6. REGULATION OF FIBROBLAST IGFBPs**  
**BY INTERLEUKIN 1 AND 6.**



## **OVERVIEW**

Although both interleukin  $1\alpha$  (IL- $1\alpha$ ) and  $1\beta$  (IL- $1\beta$ ) are biochemically and immunologically distinct from TNF $\alpha$ , and bind to different cell surface receptors, there is striking similarity in their biological activities, many of which are important in the acute inflammatory response. In fibroblasts, the cell type under investigation in this study, these shared actions include the stimulation of mitogenesis, and synthesis of procollagen, collagenase and tissue inhibitor of metalloproteinases (TIMP). As well as sharing many functions in cellular immune responses, both IL-1 and TNF- $\alpha$  can also induce the production of each other in certain other cell types, whilst the cellular signal transduction by these cytokines is also closely related, often involving certain novel serine/threonine protein kinases and cAMP, or via intermediaries such as prostaglandin E2.

Some studies have also described an interaction between IL-1 and the IGFs; such as Linkhart and MacCharles (1992) who found increased IGF-I mRNA synthesis in mouse bone calvaria in response to both IL- $1\alpha$  and IL- $1\beta$ . However, such action by IL-1 appears to be cell type specific, as Lin *et al* (1992) found the opposite effect on IGF-I mRNA in Leydig cells. The effect of these cytokines on the IGFBPs has not been investigated. In view of the functional similarities of IL-1 with TNF $\alpha$ , and the apparent modulatory role of TNF $\alpha$  in regulating fibroblast IGFBP secretion described in the previous chapter, we have investigated the effects of IL- $1\alpha$  and IL- $1\beta$  in this model system. Experiments were also undertaken to establish the effects of interleukin 6, a cytokine with rather more independent function.

## **6.1. METHODS**

### **6.1.i. Cell culture**

The normal human neonatal skin fibroblast cell line, Hs68, was used for these experiments and was routinely cultured as previously described.

### **6.1.ii. Conditioned medium**

Collection of conditioned medium from Hs68 fibroblasts followed the protocol as described in the previous chapter. For these experiments cells were seeded into 24-well tissue culture plates in complete growth medium at a density of  $1 \times 10^5$  / ml, or  $2.5 \times 10^4$

/ cm<sup>2</sup>, and incubated at 37°C for 96h. Following a further 24h in SFM, the cells were washed and the interleukin samples added for 24h.

#### **6.1.iii. Western ligand blotting (WLB)**

IGFBPs in the above CM were analysed by WLB, as described in the Methods section. For these experiments, pooled CM from triplicate wells was lyophilised under vacuum and concentrated five-fold before being loaded onto the gel.

#### **6.1.iv. IGFBP-3 radioimmunoassay**

IGFBP-3 in the conditioned medium was quantified by specific RIA, as described previously, using the rabbit polyclonal antiserum designated SCH 2/5 raised against the recombinant glycosylated IGFBP-3 peptide supplied by Celtrix Inc., Santa Clara, USA. A 50 µl volume of CM was assayed directly.

#### **6.1.v. Analysis of IGFBP gene expression by Northern blotting**

Hs68 fibroblast IGFBP-3 and IGFBP-4 gene expression in response to 24 hour incubations with 1 µg/l IL-1β or IL-6 was analysed by Northern blotting. Cells were plated into 9.5cm diameter culture dishes in a volume of 10ml and at a density of 1 x 10<sup>5</sup>/ml, or 1.3 x 10<sup>4</sup>/cm<sup>2</sup>. Following 96 hours in complete medium the cells, now approximately 80% confluent, were incubated with serum-free medium for 24 hours, washed, and finally incubated with IL-1β or IL-6. RNA isolation and Northern blotting was performed as described in the Methods section.

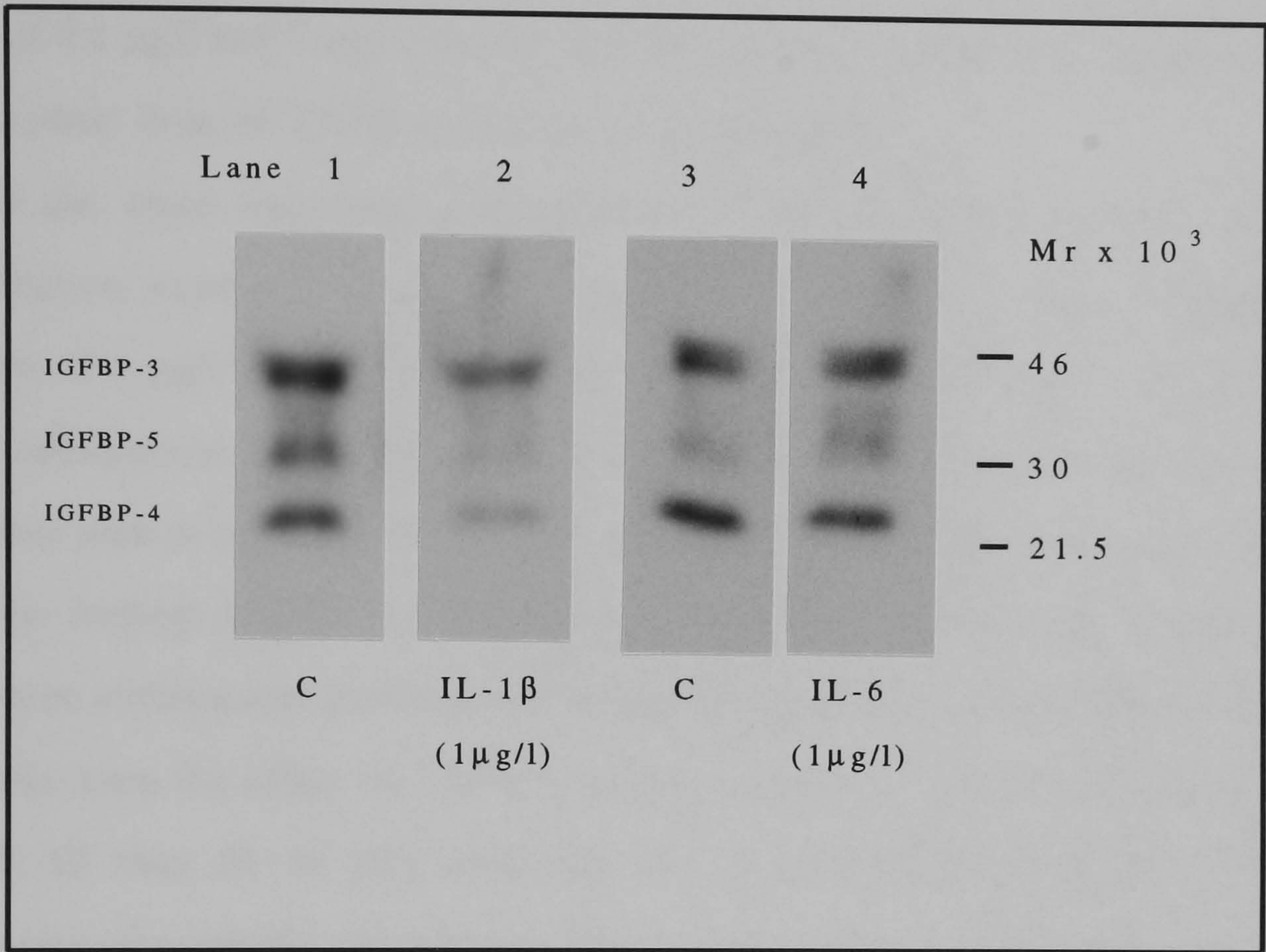
### **6.2. RESULTS - Interleukin 1**

#### **6.2.i. Western ligand blotting**

The effects of interleukin 1β on the appearance of IGFBPs in Hs68 fibroblast conditioned medium is shown in **figure 6.i**. At a dose of 1 µg/l IL-1β, changes to IGFBP-3 were observed, with a reduction in its band intensity (lane 2) compared to that of the control (lane 1). Scanning densitometric analysis of the blot revealed the IGFBP-3 level to be some 29.2% lower than control.



Figure 6.i.



The effect of IL-1 $\beta$  (1 $\mu$ g/l) and IL-6 (1 $\mu$ g/l) on IGFBP abundance in 24 hour serum-free conditioned medium taken from Hs68 fibroblasts, as assessed by Western ligand blotting. Lane 1 shows control, Lane 2 shows IL-1 $\beta$ , Lane 3 shows control and Lane 4 shows IL-6 treatment. Molecular weight standards are indicated.





### 6.2.ii. IGFBP-3 immunoreactivity

IGFBP-3 radioimmunoassay analysis of Hs68 cell conditioned medium following IL-1 $\beta$  treatment, as shown in **figure 6.ii.**, demonstrated that the cytokine inhibited the secretion of the binding protein in a dose-dependent manner. The effective doses and degree of IGFBP-3 response to IL-1 $\beta$  were similar to TNF $\alpha$ , with significant inhibition at levels of IL-1 $\beta$  of 0.1  $\mu$ g/l and 1  $\mu$ g/l causing a 31.8% reduction in IGFBP-3 compared with 27.6% for the same dose of TNF $\alpha$  in this set of experiments.

Due to the close functional association of IL-1 $\beta$  and TNF $\alpha$  in many other systems, coincubation experiments were also performed. As can be seen in **figure 6.iii.**, the addition of 1  $\mu$ g/l IL-1 $\beta$  to the effective dose-range of TNF $\alpha$  (0.1 - 10  $\mu$ g/l) resulted in slight enhancement of IGFBP-3 inhibition. Significant alteration in the level of IGFBP-3 from that seen in response to IL-1 $\beta$  alone was observed in the presence of 10  $\mu$ g/l TNF $\alpha$  only, the highest dose tested, but was less than additive. This may suggest perhaps that a common exhaustable pathway was being utilised by the two cytokines, although as we have also seen the effect of TNF $\alpha$  to plateau between 1 and 10  $\mu$ g/l we cannot discount that IL-1 $\beta$  may be in part operating via an alternative mechanism. Unfortunately, constraints of available recombinant TNF $\alpha$  made further experiments with higher TNF $\alpha$  doses impossible.

The effects of IL-1 $\alpha$  on IGFBP-3 secretion were also assessed by radioimmunoassay. As shown in **figure 6.iv.**, this cytokine was equally effective at inhibiting Hs68 cell IGFBP-3 secretion as the functionally identical IL-1 $\beta$ , with similar effective doses and degree of response.

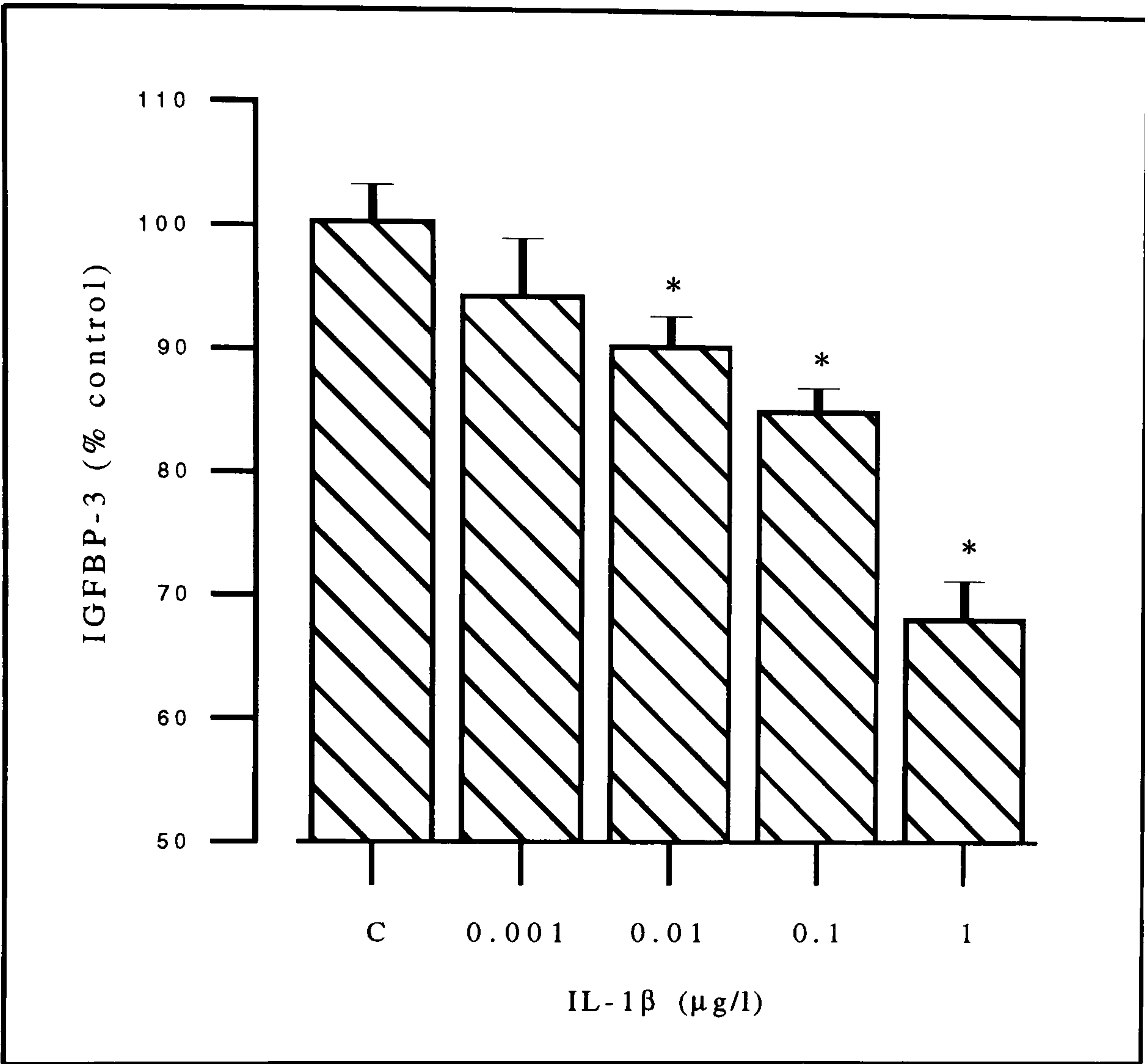
### 6.2.iii. IGFBP gene expression

#### IGFBP-3

Despite the close similarity in IGFBP-3 peptide secretion following treatment with IL-1 $\beta$  or TNF $\alpha$ , differences were observed in their effect upon IGFBP-3 gene expression. Unlike TNF $\alpha$ , which had no inhibitory effect on IGFBP-3 mRNA abundance after 24 hours of treatment, 1  $\mu$ g/l IL-1 $\beta$  caused a reduction in the single 2.4 kb transcript, as shown in **figure 6.v.** Scanning densitometry showed the decrease to be a 30% reduction from the unstimulated control. These data suggest therefore that IL-1 $\beta$  mediated inhibition of



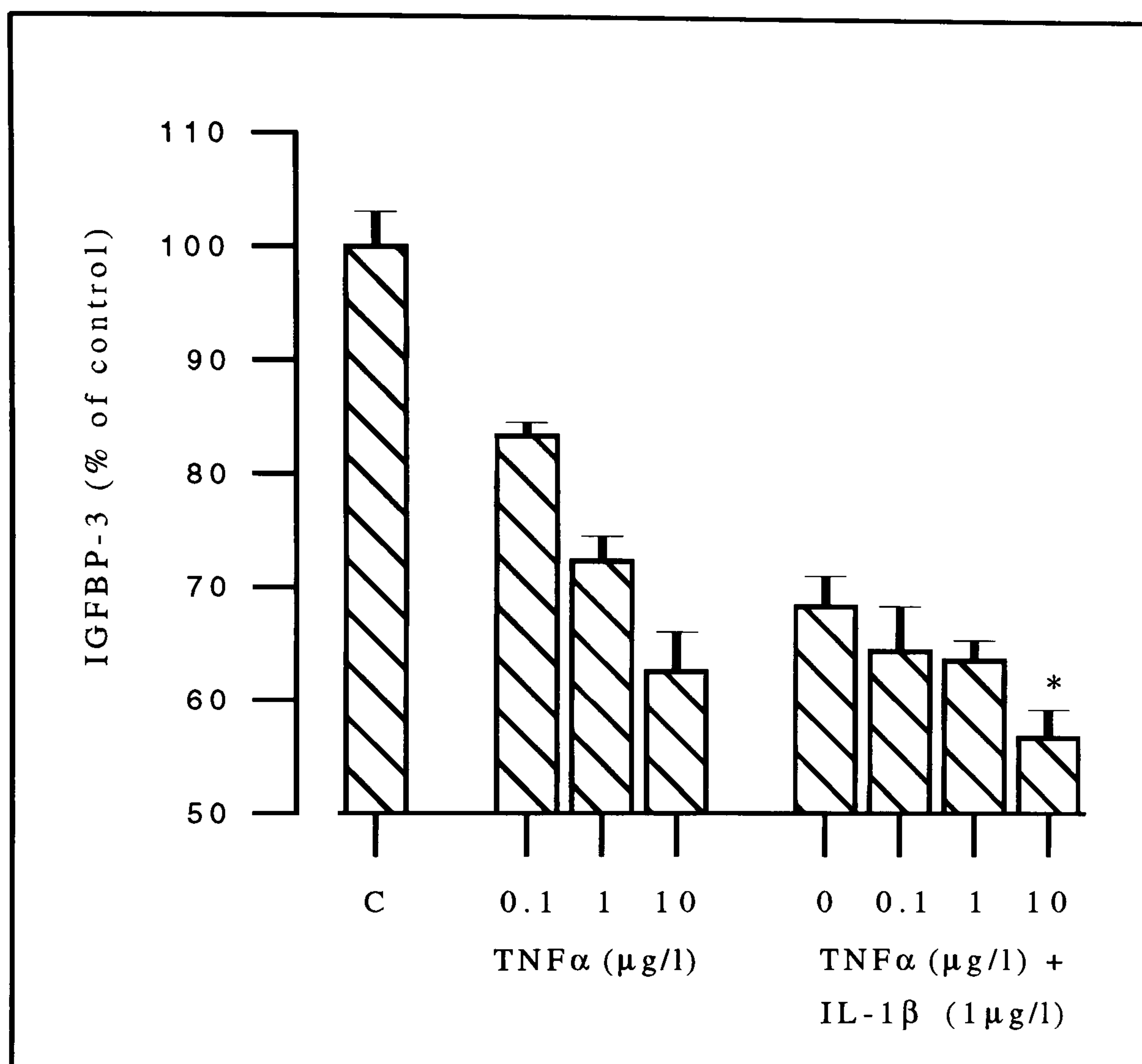
Figure 6.ii.



Dose-dependent inhibition of immunoreactive IGFBP-3 secretion from Hs68 fibroblasts by interleukin-1 $\beta$ . Results are expressed as the percentage IGFBP-3 in 24 hour conditioned medium compared to the basal serum-free control  $\pm$  S.D.

\* P<0.05 compared to control.

Figure 6.iii.

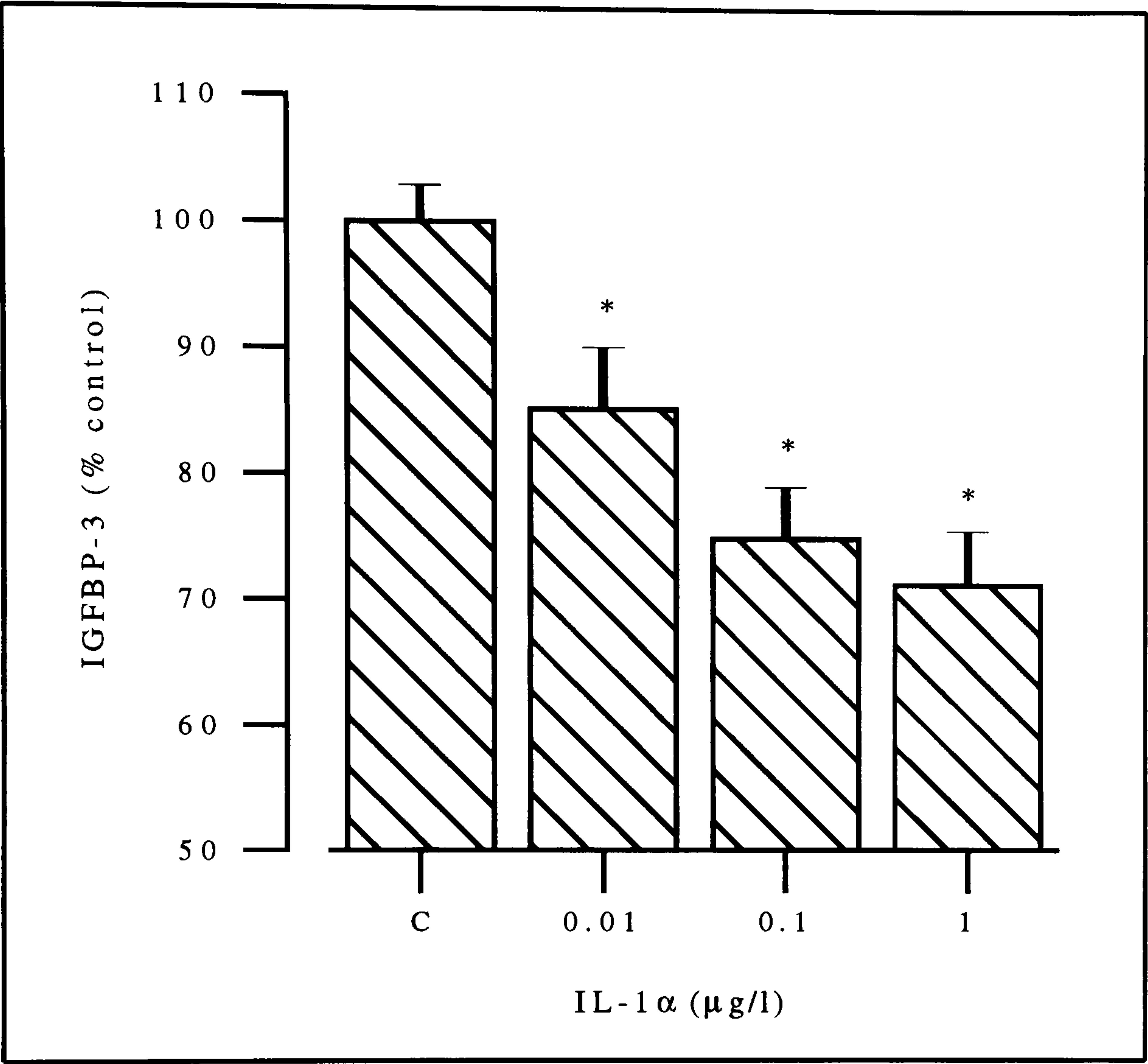


The effect of IL-1 $\beta$  (1 $\mu$ g/l) on the inhibition by TNF $\alpha$  of immunoreactive IGFBP-3 in 24 hour serum-free conditioned medium from Hs68 fibroblasts. Results from triplicate cultures are expressed as the percentage IGFBP-3 compared to the untreated control value  $\pm$  S.D.

\* - P<0.05 compared to IL-1 $\beta$  alone.



Figure 6.iv.

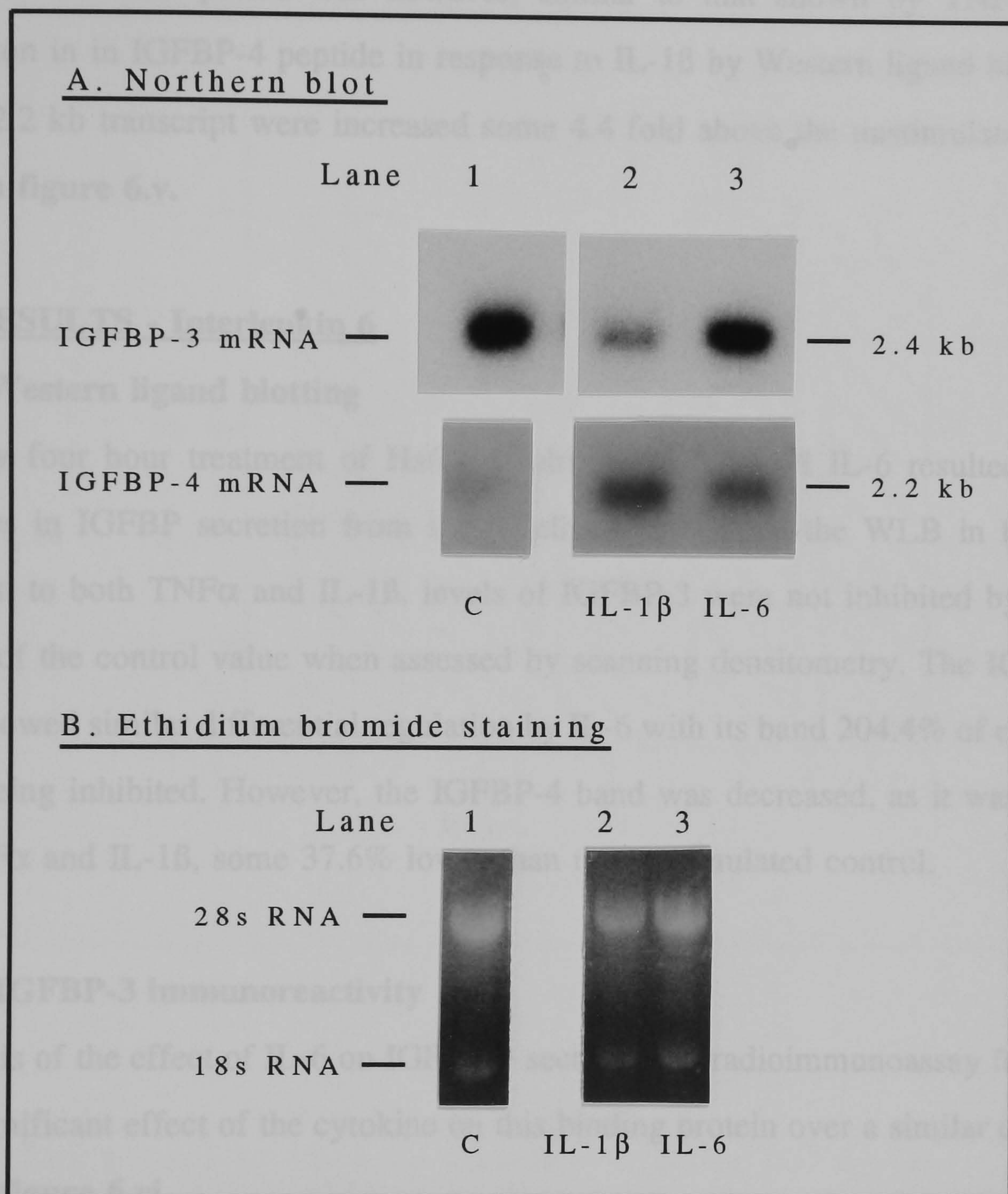


Dose-dependent inhibition of immunoreactive IGFBP-3 secretion from Hs68 fibroblasts by interleukin-1 $\alpha$ . Results are expressed as the percentage IGFBP-3 in 24 hour conditioned medium compared to the basal serum-free control  $\pm$  S.D.

\* P<0.05 compared to control.



Figure 6.v.



Regulation of IGFBP-3 and IGFBP-4 gene expression in Hs68 fibroblasts by 1μg/l IL-1β and 1μg/l IL-6. Shown in panel A are IGFBP-3 and -4 mRNA levels following 24 hours of treatment. In panel B ethidium bromide staining of the gel is shown to confirm that similar amounts of total RNA were loaded into each lane.



IGFBP-3 peptide may occur via down-regulated gene expression.

#### IGFBP-4

The effect of IL-1 $\beta$  (1  $\mu$ g/l) upon Hs68 cell IGFBP-4 gene expression at the end of the 24 hour treatment period was however, similar to that shown by TNF $\alpha$ . Despite a reduction in IGFBP-4 peptide in response to IL-1 $\beta$  by Western ligand blotting, levels of its 2.2 kb transcript were increased some 4.4 fold above the unstimulated control, as seen in **figure 6.v**.

### **6.3. RESULTS - Interleukin 6**

#### **6.3.i. Western ligand blotting**

Twenty four hour treatment of Hs68 fibroblasts with 1  $\mu$ g/l IL-6 resulted in minimal changes in IGFBP secretion from these cells, as shown in the WLB in **figure 6.i**. In contrast to both TNF $\alpha$  and IL-1 $\beta$ , levels of IGFBP-3 were not inhibited by IL-6, being 135% of the control value when assessed by scanning densitometry. The IGFBP-5 band also showed similar differential regulation by IL-6 with its band 204.4% of control, rather than being inhibited. However, the IGFBP-4 band was decreased, as it was in response to TNF $\alpha$  and IL-1 $\beta$ , some 37.6% lower than the unstimulated control.

#### **6.3.ii. IGFBP-3 immunoreactivity**

Analysis of the effect of IL-6 on IGFBP-3 secretion by radioimmunoassay failed to show any significant effect of the cytokine on this binding protein over a similar dose-range as IL-1 $\beta$ ; **figure 6.vi**.

#### **3.3.vi. IGFBP gene expression**

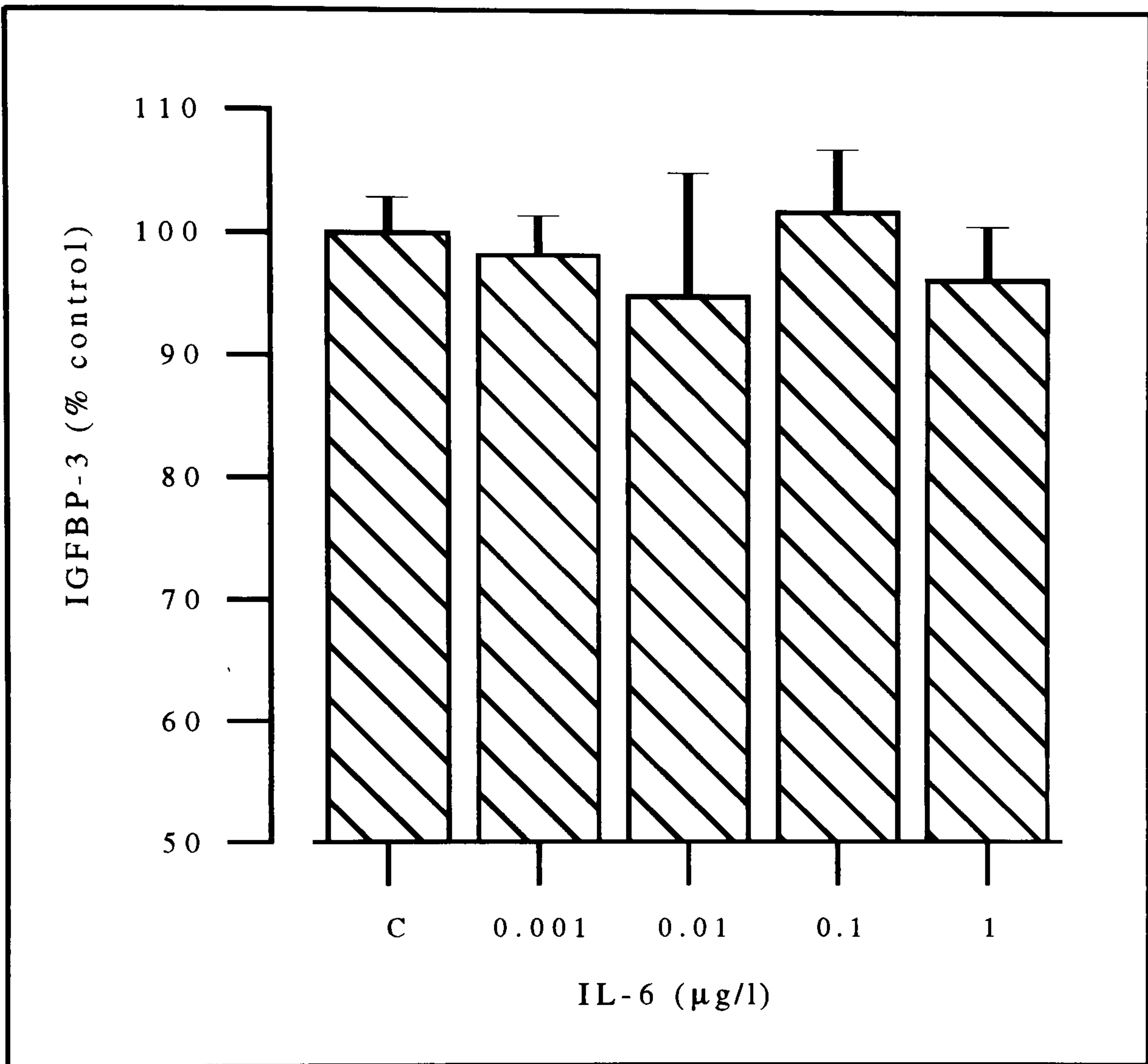
##### IGFBP-3

As shown in **figure 6.v.**, a dose of 1  $\mu$ g/l IL-6 had minimal effect upon IGFBP-3 mRNA abundance at the end of the 24 hour treatment period.

##### IGFBP-4

In a similar manner to both TNF $\alpha$  and IL-1 $\beta$ , IL-6 (1  $\mu$ g/l) lead to a small increase in the

Figure 6.vi.



The effect of IL-6 on immunoreactive IGFBP-3 secretion from Hs68 fibroblasts. Results are expressed as the percentage IGFBP-3 in 24 hour conditioned medium compared to the untreated serum-free control  $\pm$  S.D.



abundance of the 2.2 kb IGFBP-4 mRNA transcript after 24 hours of treatment. Scanning densitometry of the autoradiograph shown in **figure 6.v.** gave this increase as 152% above the control value.

#### **6.4. CONCLUSIONS**

A high degree of functional similarity between TNF $\alpha$  and IL-1 has been described in a number of biological systems, and it would appear from our data that this relationship also extends to their effects upon IGFBP secretion *in vitro*. The inhibitory nature seen by WLB analyses of IL-1 $\beta$  on IGFBP-3, IGFBP-4 and IGFBP-5 levels in 24 hour conditioned media from Hs68 fibroblasts parallels that seen in response to TNF $\alpha$ . Further investigation of IGFBP-3 secretion from these cells by RIA demonstrated that the two cytokines were active over the same concentration range and inhibited the binding protein to a similar degree. The functionally identical, but structurally distinct IL-1 species, IL-1 $\alpha$ , also similarly inhibited immunoreactive IGFBP-3 levels.

Although TNF $\alpha$  and IL-1 share many biological properties, the precise mechanisms involved in this relationship are unclear. The activation of common signal transduction pathways, possibly involving novel protein kinases (Guy *et al*, 1991), in both of the respective receptors may provide some explanation, although there are other studies that suggest some of the precise molecular actions of IL-1 and TNF $\alpha$  may in fact be different. Evidence for such differential mechanisms of action has been given as the often observed synergistic relationship between the two cytokines (Ruggiero and Baglioni, 1987), and from IL-1 antagonism of TNF $\alpha$  cytotoxicity seen in some cells (Holtman and Wallach, 1987). Co-incubation of IL-1 $\beta$  with TNF $\alpha$  in our model produced no synergistic effect and was not antagonistic, perhaps suggesting the use of a common mechanism in their regulation of IGFBP-3.

Unlike TNF $\alpha$  however, the abundance of IGFBP-3 mRNA in Hs68 fibroblasts was reduced at the end of the 24 hour treatment period with IL-1 $\beta$ , suggesting that altered gene expression may be at least partly involved in mediating the inhibition of IGFBP-3 peptide. The similar discordant IGFBP-4 mRNA response to both IL-1 $\beta$  and TNF $\alpha$ , where

the reduction in IGFBP-4 peptide was accompanied by increased gene expression at the end of the incubation period, may indicate a common mechanism of action between the cytokines in modulating this particular IGFBP species.

In contrast to the effects of the two closely related cytokines IL-1 and TNF $\alpha$ , we found no evidence that IL-6 had any impact on IGFBP secretion from Hs68 fibroblasts. The lack of an effect of IL-6 on IGFBP production suggests cytokine specificity in the IGFBP response, with alteration in IGFBPs only occurring during inflammatory episodes involving IL-1 and TNF $\alpha$ .



**CHAPTER 7. REGULATION OF FIBROBLAST IGFBPs**  
**BY TRANSFORMING GROWTH FACTOR  $\beta_1$**

## **OVERVIEW**

The IGFs have been widely recognised as having a major role in the processes of wound tissue repair. At sites of injury platelets rapidly congregate, adhere to exposed collagen and release their secretory alpha-granules, containing numerous growth factors, such as the IGFs. Other IGFs from plasma and those secreted from invading macrophages are also present, resulting in typical wound site levels reaching approximately two thirds of that found in serum. The physiological importance of these high IGF levels has been demonstrated experimentally (Sommer *et al*, 1991) with their inhibition resulting in a 40% reduction in wound collagen production.

Another cytokine or growth factor to be implicitly involved in the repair of wound tissue is transforming growth factor  $\beta$  (TGF $\beta$ ). Many of its recognised functions seem particularly directed towards the wound response, such as its regulation of mitogenesis, the stimulation of extracellular matrix (both directly and by inhibiting destructive metalloproteinases) and induction of chemotactic migration. A recent study by Baxter *et al* (1991) has also shown this cytokine to be capable of enhancing IGFBP-3 secretion by human skin fibroblasts, and therefore may have the potential to mediate IGF bioactivity. In view of the data presented in the previous chapters concerning the apparent down-regulation of Hs68 cell IGFBPs, including IGFBP-3, by TNF $\alpha$  and certain of the interleukins, we have further investigated TGF $\beta$  in our model. By comparing and contrasting the effects and mechanisms of TGF $\beta$  with the inflammatory cytokines we hoped some insight into their respective functions as mediators of cellular growth would be gained.

## **7.1. METHODS**

### **7.1.i. Cell culture**

The normal human neonatal skin fibroblast cell line, Hs68, was routinely cultured as previously described.

### **7.1.ii. Conditioned medium**

Cells were seeded into 24-well tissue culture plates in complete growth medium (10% foetal calf serum) in a volume of 0.5 ml / well, and at densities of 0.5, 1, 2.5 and 10 x



$10^4$  / ml or 0.125, 0.250, 0.625 and  $2.5 \times 10^4$  /  $\text{cm}^2$ , respectively. This range of densities allowed analysis of the effects of TGF $\beta$ <sub>1</sub> on fibroblast cultures from sparse to confluent populations.

Following the culture protocol described in the previous chapter, conditioned medium (CM) from these cells was collected after 24 hour incubations with human recombinant TGF $\beta$ <sub>1</sub> over a dose range of 0.01 to 10  $\mu\text{g/l}$ .

#### **7.1.iii. Western ligand blotting (WLB)**

IGFBPs in the above CM were analysed by WLB, based upon that of Hossenlopp *et al* (1986) and as described in the Methods chapter. Specifically for these set of experiments, pooled CM from triplicate wells was dried under vacuum and concentrated five-fold before being loaded onto the gel.

#### **7.1.iv. IGFBP-3 radioimmunoassay**

IGFBP-3 in the CM was quantified by specific RIA, as described previously, using the rabbit polyclonal antiserum designated 1287-2-14 from Celtrix Inc., Santa Clara, USA. Due to the sensitivity of this RIA no concentration of CM was necessary, with 50  $\mu\text{l}$  measured directly.

The effect of fibronectin upon the TGF $\beta$ <sub>1</sub> enhancement of IGFBP-3 was also studied by radioimmunoassay. Reasoning behind this was due to a study by Ignatz and Massague (1987) that had previously shown that the effect of TGF $\beta$  on inducing anchorage-independent growth of fibroblasts was due to increased synthesis of this extracellular matrix component and could be mimicked by the addition of exogenous fibronectin. Both could be blocked by the addition of short synthetic peptides containing RGD (Arg-Gly-Asp) amino acid sequences, which inhibit binding of the fibronectin to cell membrane integrin receptors.

#### **7.1.v. Northern blotting**

Hs68 IGFBP-3 gene expression in response to 24h incubations with 1  $\mu\text{g/l}$  TGF $\beta$ <sub>1</sub> was analysed by Northern blotting. Cells were plated into 9.5cm tissue culture dishes in a volume of 10ml and at a density of  $1 \times 10^5/\text{ml}$ , or  $1.3 \times 10^4/\text{cm}^2$ . Such an initial density



meant that cultures were sub-confluent (approximately 75%) after 96 hours in complete growth medium. They were then incubated for 24h in SFM, washed and finally incubated with TGFβ<sub>1</sub>. RNA isolation and Northern blotting was undertaken as detailed in the Methods chapter.

#### **7.1.vi. IGFBP protease analysis**

In view of the well documented role that TGFβ has in modulating the activity of certain metalloproteinases *in vitro*, experiments were performed to examine its effect upon the recently identified IGFBP protease(s). Conditioned medium was taken from Hs68 fibroblast cells following either serum-free treatment alone or treatment with 1 µg/l TGFβ<sub>1</sub> for 24 hours, and incubated with <sup>125</sup>I-IGFBP-3 for 4 hours at 37°C. Alteration in proteolytic activity directed against this peptide was assessed by subsequent polyacrylamide gel electrophoresis as described in the Methods section.

### **7.2. RESULTS**

#### **7.2.i. Western ligand blotting**

As described in **figure 7.i.**, 24 hour treatment of Hs68 fibroblasts with 1 µg/l TGFβ<sub>1</sub> resulted in changes to all the IGFBPs produced by these cells when assessed by WLB. Scanning densitometric analysis of the autoradiograph revealed IGFBP-3 band to be 341% of the serum-free control, whilst IGFBP-4 and IGFBP-5 were in contrast reduced by 61% and 49.8%, respectively.

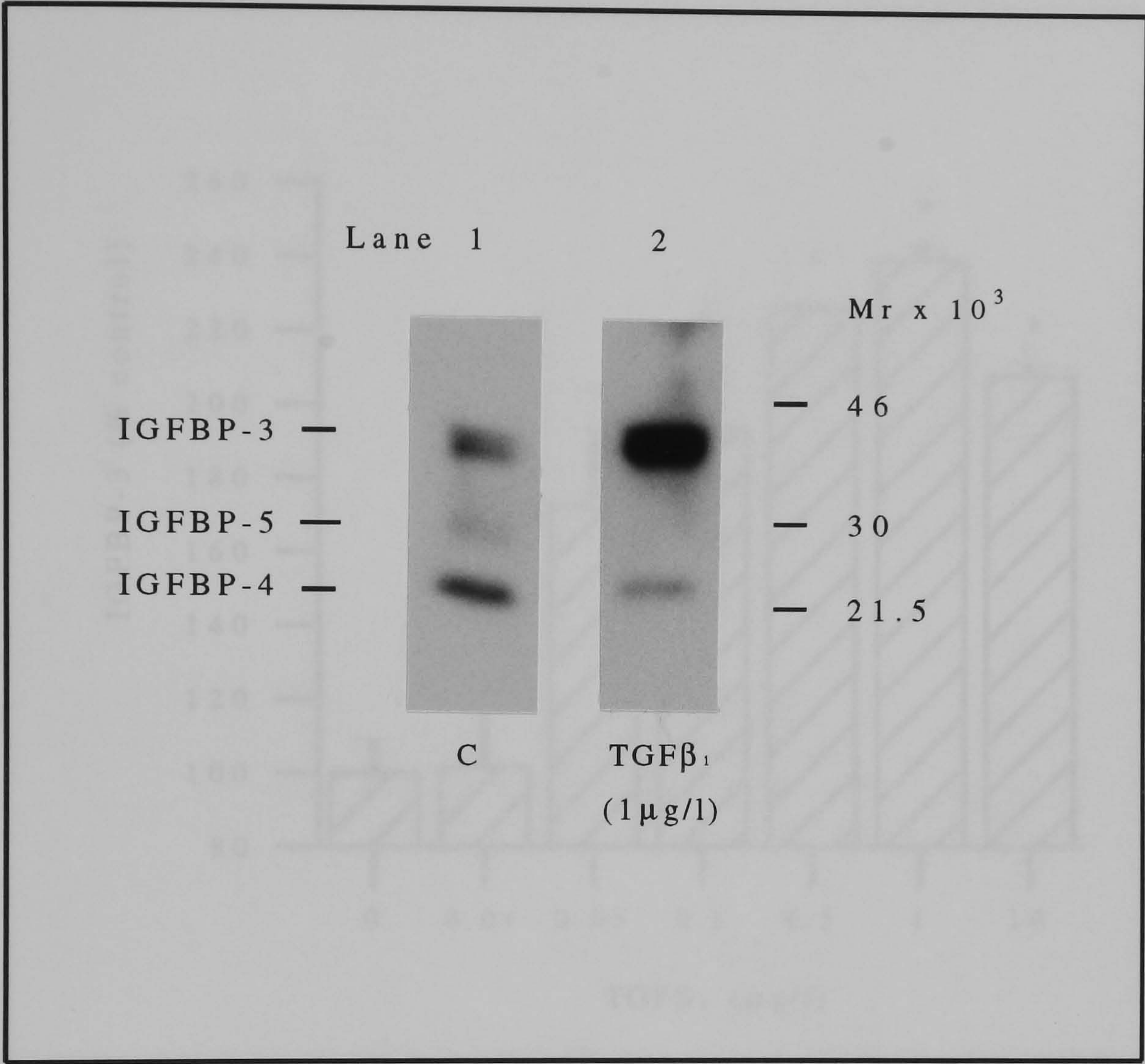
#### **7.2.ii. IGFBP-3 immunoreactivity**

The stimulating effect of TGFβ<sub>1</sub> upon the concentration of IGFBP-3 in the conditioned medium from Hs68 cells was further assessed by radioimmunoassay as described in **figure 7.ii.** Significant stimulation of IGFBP-3 was observed at TGFβ<sub>1</sub> doses of 0.05 µg/l and above, and whilst a dose-dependent effect was generally observed a small hook effect was seen at the highest dose tested of 10 µg/l.

Further investigation of the effect highlighted its dependency upon cell density, as shown in **figure 7.iii.** The degree of IGFBP-3 stimulation was greatest, 260.8 ± 22.4% of control, in the most sparsely plated cultures (0.5 x 10<sup>4</sup>/ml) whilst in fully confluent monolayers



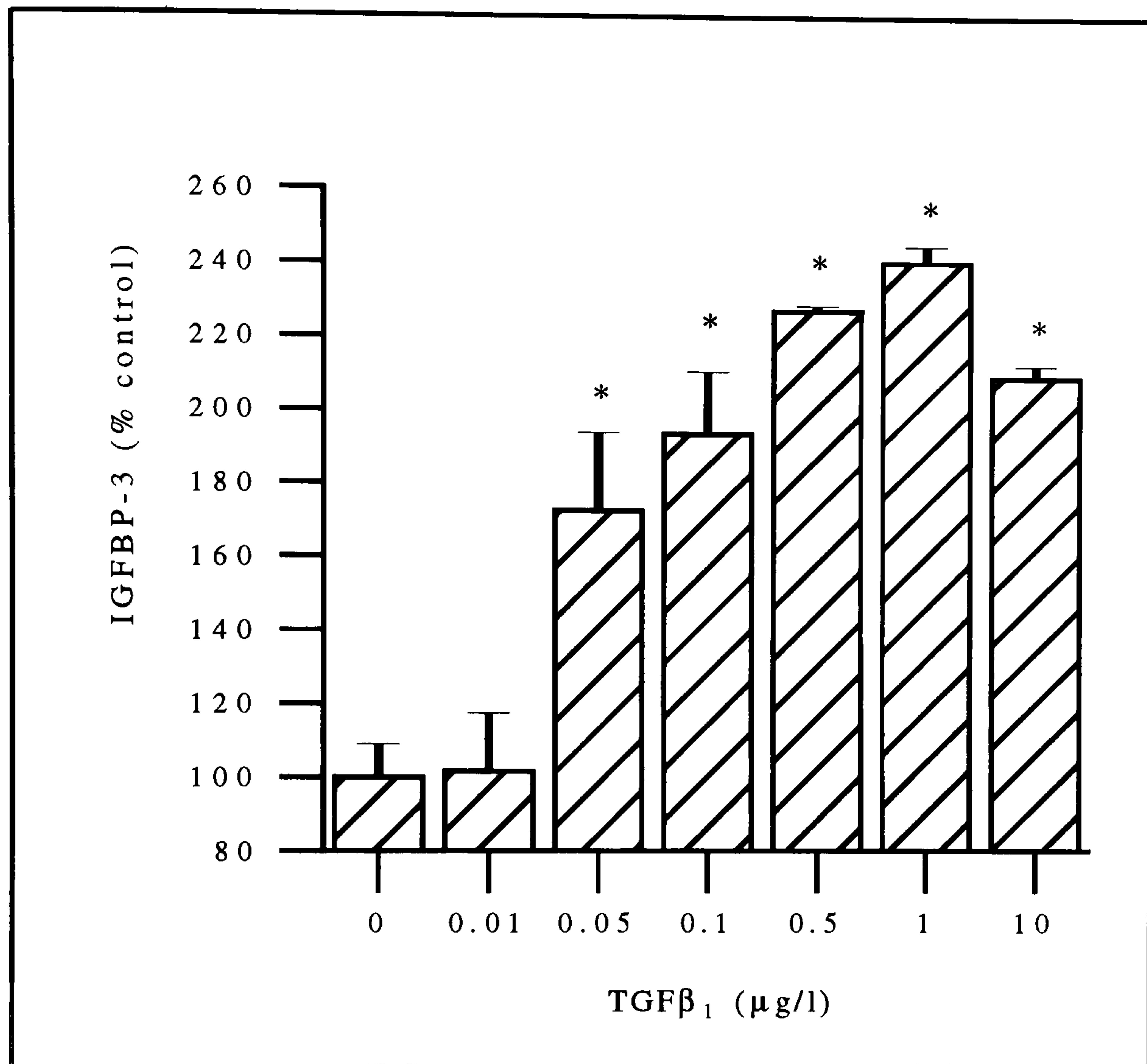
Figure 7.i.



The effect of  $1\mu\text{g/l}$   $\text{TGF}\beta_1$  on IGFBP abundance in 24 hour serum-free conditioned medium taken from Hs68 fibroblasts, as assessed by Western ligand blotting. Lane 1 shows control, Lane 2 shows  $\text{TGF}\beta_1$  treatment. Molecular weight standards are indicated.



Figure 7.ii.

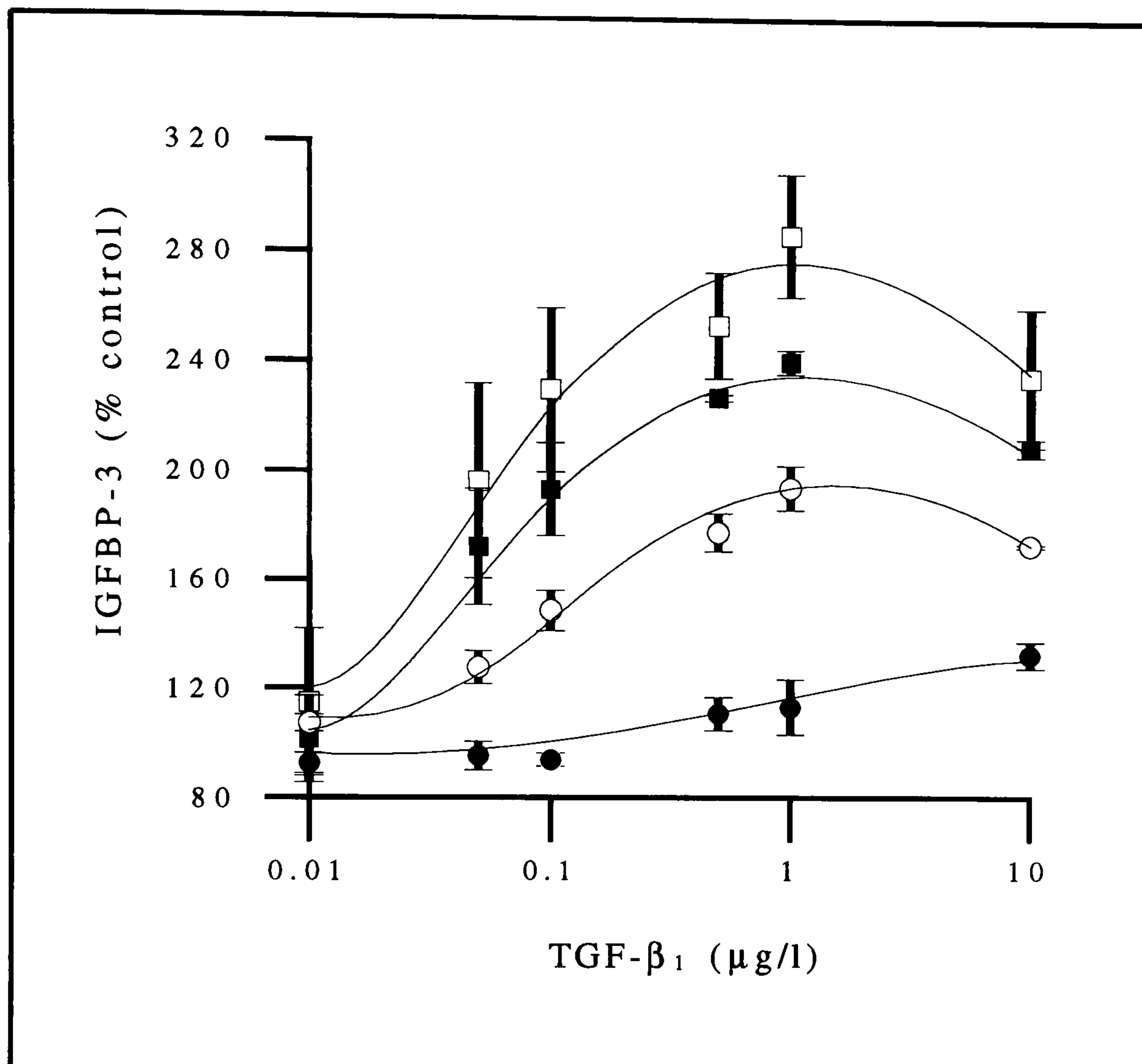


The effect of transforming growth factor  $\beta_1$  (TGF $\beta_1$ ) on immunoreactive IGFBP-3 levels in serum-free medium conditioned by Hs68 cells for 24 hours. Cells were plated at a density of  $1 \times 10^4$ /ml, and IGFBP-3 measured by radioimmunoassay as described in the Methods. Results are expressed as percentage IGFBP-3 of the serum-free control  $\pm$  S.D.

\*  $P < 0.05$  compared with control.



Figure 7.iii.



The effect of cell density on TGF $\beta_1$  mediated stimulation of IGFBP-3 secretion from Hs68 cells. Cells were plated at 2.5 ( $\square$ ) and 5  $\times 10^3$ /well ( $\blacksquare$ ), 1.25 ( $\circ$ ) and 5  $\times 10^4$ /well ( $\bullet$ ) before exposure to TGF $\beta_1$  (1 $\mu\text{g/l}$ ) for 24 hours. IGFBP-3 was quantified by radioimmunoassay and results expressed as the percentage IGFBP-3 of serum-free controls  $\pm$  S.D.

( $1 \times 10^5$ /ml) significant stimulation of IGFBP-3 was only observed at a  $\text{TGF}\beta_1$  dose of 1  $\mu\text{g/l}$  or more, achieving a modest increase of  $32.6 \pm 4.9\%$  above control at 10  $\mu\text{g/l}$ .

To establish whether enhanced IGFBP-3 secretion by  $\text{TGF}\beta_1$  was a direct function or secondary to stimulation of Hs68 cell fibronectin, experiments were performed to study in this model the effects of exogenous recombinant bovine fibronectin and synthetic RGD sequences. Using doses comparable with those found effective in the Ignatz and Massague study, 24 hour treatment of cells with fibronectin alone (50 mg/l) or in combination with  $\text{TGF}\beta_1$  (1  $\mu\text{g/l}$ ) had no significant effects upon IGFBP-3 secretion, when assessed by RIA (**figure 7.iv.**). In addition, the presence of the RGD sequence peptide (400 mg/l) had no inhibitory effect on either basal or  $\text{TGF}\beta_1$  enhanced IGFBP-3 secretion in these cells.

### **7.2.iii. IGFBP gene expression**

#### **IGFBP-3**

Northern blotting of total RNA from Hs68 cells following 24 hour treatment with  $\text{TGF}\beta_1$  (1  $\mu\text{g/l}$ ) revealed that enhancement of IGFBP-3 peptide secretion was associated with increased IGFBP-3 gene expression, as shown in **figure 7.v.** Scanning densitometric analysis of the autoradiograph showed the single 2.4kb IGFBP-3 mRNA transcript to be increased 12.7 fold over the serum-free control value. Initial loading of the RNA into the control and  $\text{TGF}\beta_1$  treatment lanes of the gel appeared similar under ultra violet light.

#### **IGFBP-4**

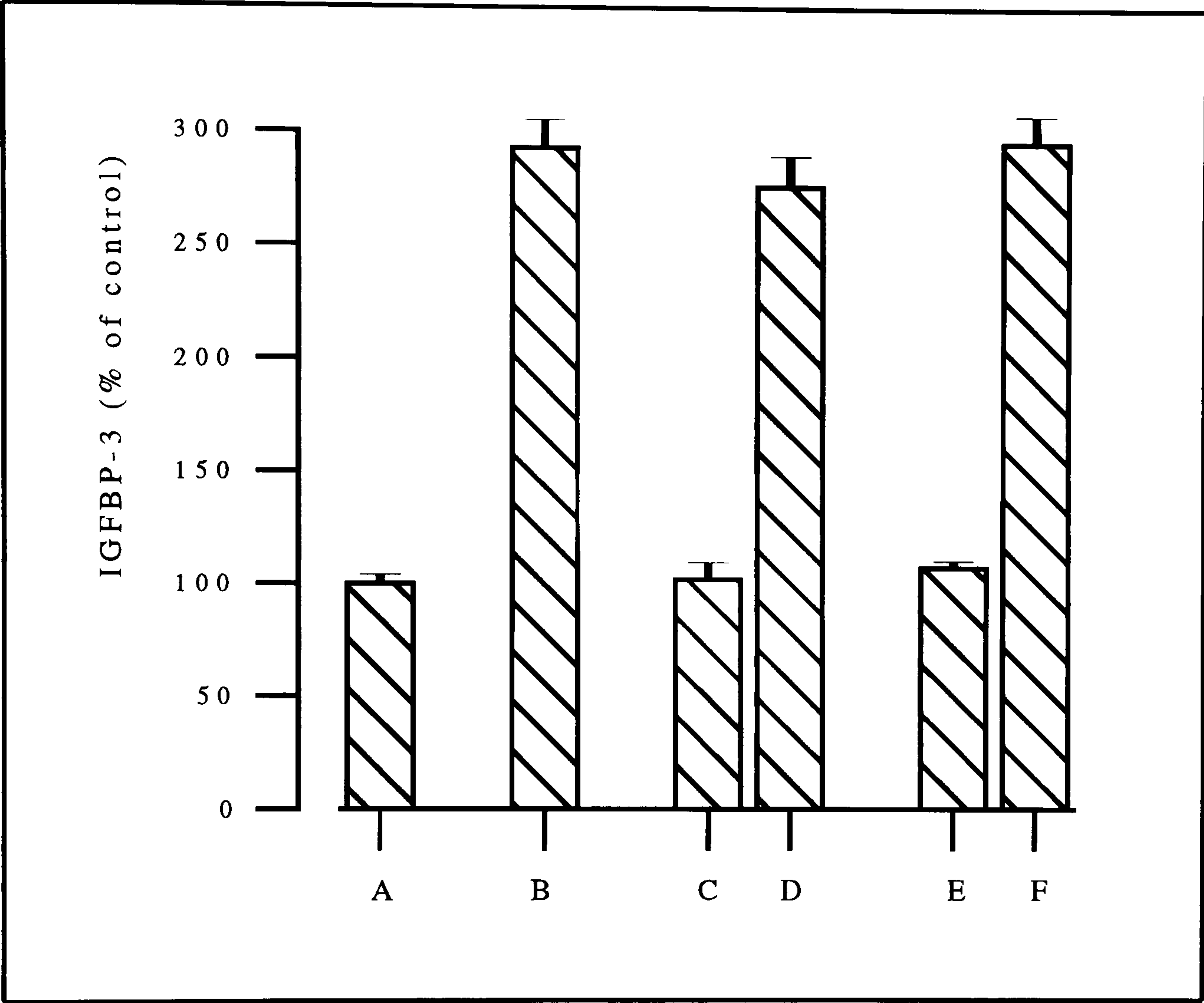
Following stringent washing, the nylon Northern blot membrane probed with the IGFBP-3 cDNA was rehybridised with a cDNA for IGFBP-4. Despite  $\text{TGF}\beta_1$  causing a reduction in IGFBP-4 peptide on WLB, no significant alteration in its gene expression was seen at the end of the 24 hour treatment period, **figure 7.v.** Scanning densitometric analysis of the  $\text{TGF}\beta_1$  treatment lane showed the single 2.2 kb transcript to be 109% of the control value.

### **4.2.iv. IGFBP-3 protease**

The effect of  $\text{TGF}\beta_1$  on proteolytic activity produced by Hs68 fibroblasts and directed



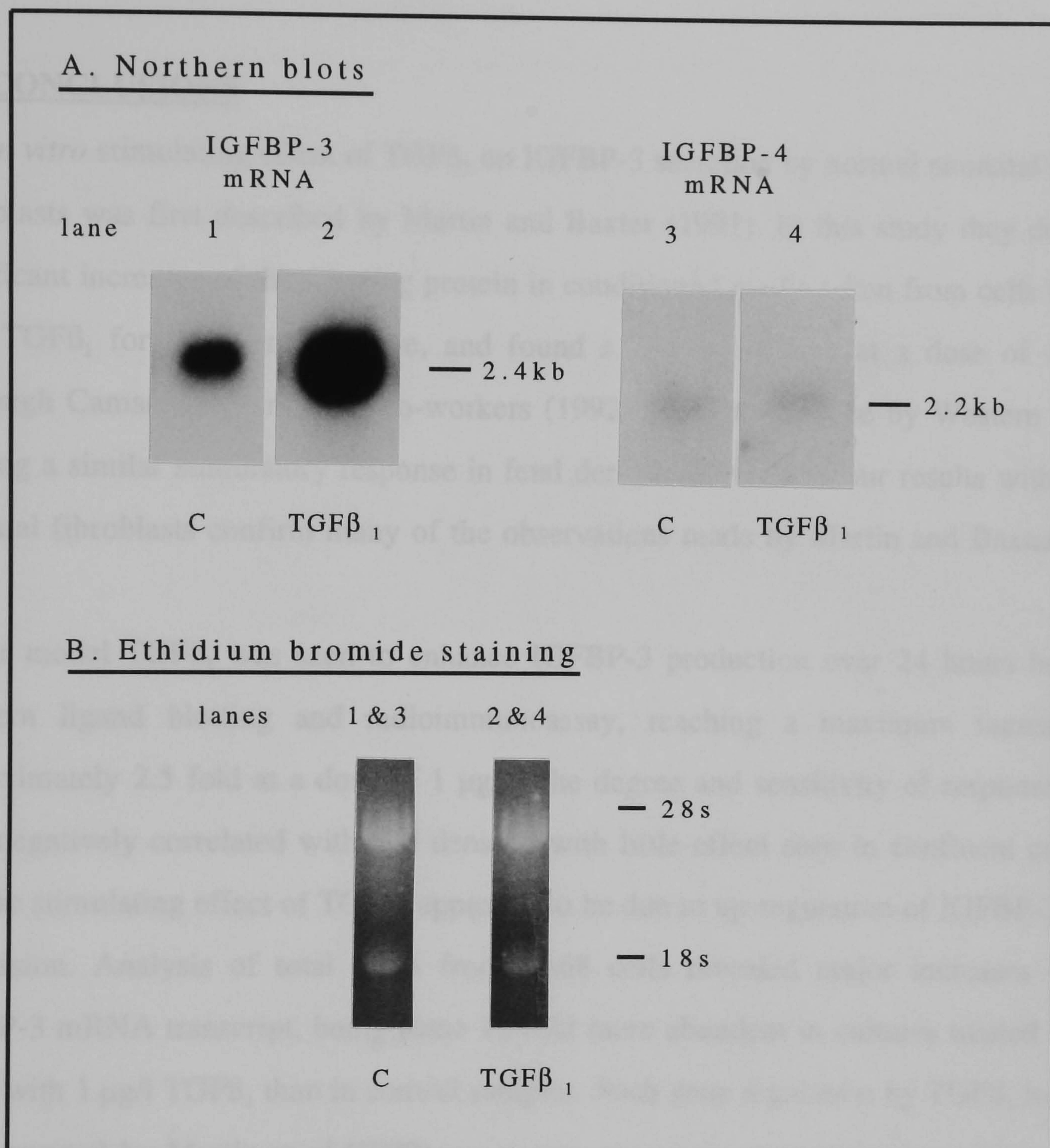
Figure 7.iv.



The effect of exogenous fibronectin and synthetic RGD amino acid peptide on basal and  $\text{TGF}\beta_1$  stimulated IGFBP-3 secretion by Hs68 fibroblasts. Subconfluent cultures were incubated for 24 hours at  $37^\circ\text{C}$  with : A) serum-free medium alone; B)  $\text{TGF}\beta_1$  ( $1\mu\text{g}/\text{l}$ ); C) fibronectin ( $50\text{mg}/\text{l}$ ); D) fibronectin +  $\text{TGF}\beta_1$ ; E) RGD peptide ( $400\text{ mg}/\text{l}$ ); and F) RGD +  $\text{TGF}\beta_1$ . IGFBP-3 was assessed by radioimmunoassay and results expressed as percentage IGFBP-3 of the serum-free control  $\pm$  S.D.



Figure 7.v.



Regulation of IGFBP-3 and IGFBP-4 gene expression in Hs68 fibroblasts by 1µg/l TGFβ<sub>1</sub>. Shown in panel (A) are IGFBP-3 and -4 mRNA levels following 24 hours of treatment at 37°C. In panel (B) the ethidium bromide staining of the 28s and 18s RNA is shown to indicate the accuracy of loading 15µg total RNA per lane.



against IGFBP-3 is shown in **figure 7.vi**. Despite being a well recognised mediator of fibroblast metalloproteinases, proteolytic degradation of  $^{125}\text{I}$ -IGFBP-3 following 4 hour incubations remained constant in conditioned media samples taken from cells treated for 24 hours with  $\text{TGF}\beta_1$  (1  $\mu\text{g/l}$ ) compared to those in serum-free conditions only.

### **7.3. CONCLUSIONS**

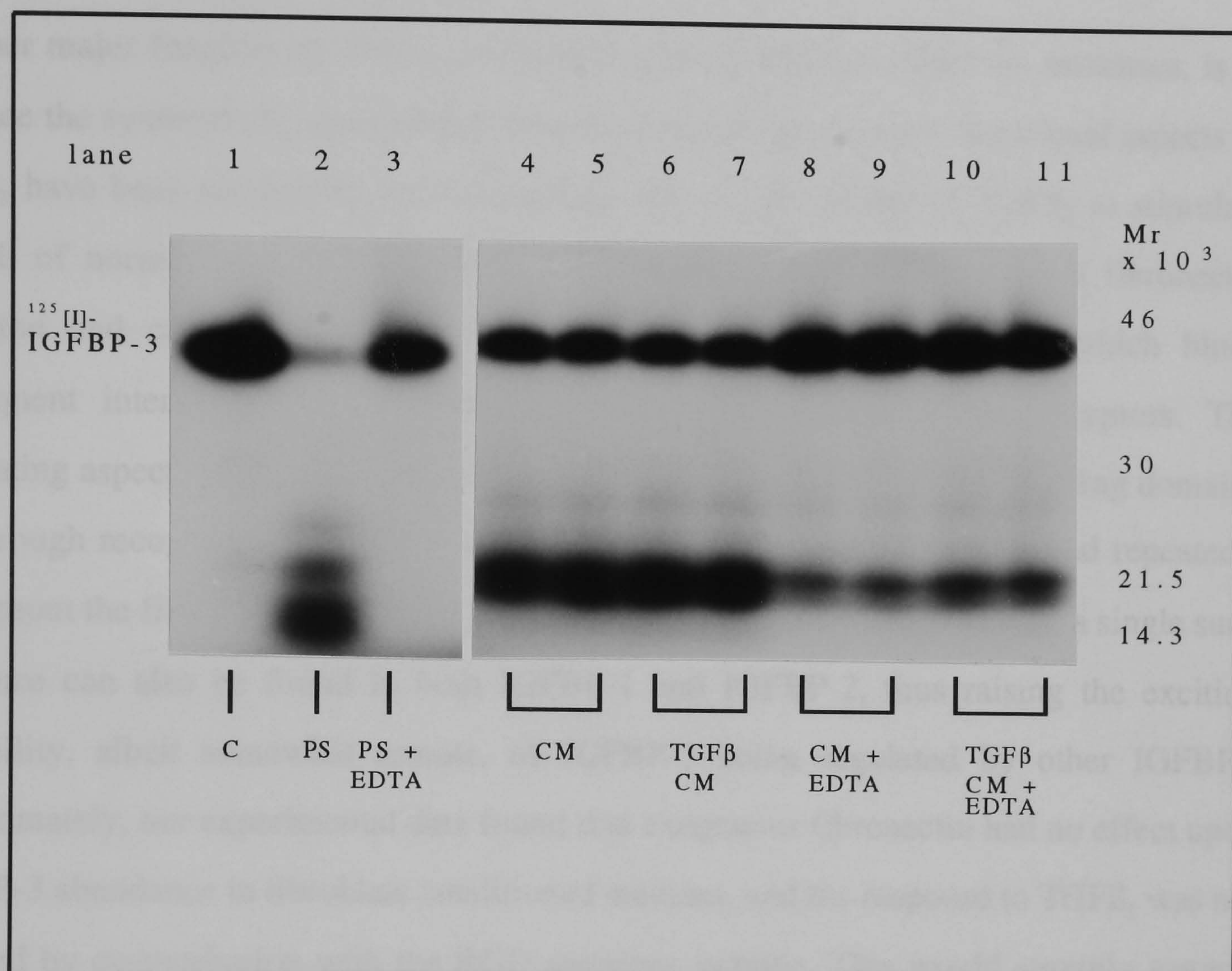
The *in vitro* stimulating effect of  $\text{TGF}\beta_1$  on IGFBP-3 secretion by normal neonatal human fibroblasts was first described by Martin and Baxter (1991). In this study they detected significant increases of this binding protein in conditioned media taken from cells treated with  $\text{TGF}\beta_1$  for 24 hours or more, and found a maximal effect at a dose of 1  $\mu\text{g/l}$ . Although Camacho-Hubner and co-workers (1992) failed to observe by Western ligand blotting a similar stimulatory response in fetal derived fibroblasts, our results with Hs68 neonatal fibroblasts confirm many of the observations made by Martin and Baxter.

In our model  $\text{TGF}\beta_1$  was seen to enhance IGFBP-3 production over 24 hours both by Western ligand blotting and radioimmunoassay, reaching a maximum increase of approximately 2.5 fold at a dose of 1  $\mu\text{g/l}$ . The degree and sensitivity of response were both negatively correlated with cell density, with little effect seen in confluent cultures and the stimulating effect of  $\text{TGF}\beta_1$  appeared to be due to up-regulation of IGFBP-3 gene expression. Analysis of total RNA from Hs68 cells revealed major increases in the IGFBP-3 mRNA transcript, being some 12-fold more abundant in cultures treated for 24 hours with 1  $\mu\text{g/l}$   $\text{TGF}\beta_1$  than in control samples. Such gene regulation by  $\text{TGF}\beta_1$  has also been reported by Martin *et al* (1992).

As  $\text{TGF}\beta_1$  is widely recognised as having an inhibitory role in cellular protease synthesis, as detailed in the Introduction, it seemed an attractive and somewhat likely mechanism to help explain its enhancing effect upon IGFBP-3, in addition perhaps to the molecular events described above. A down-regulation of collagenase synthesis, an enzyme known to be affected by  $\text{TGF}\beta_1$  (Edwards *et al*, 1987) and effective against IGFBP-3 (Frost V.J. Ph.D. thesis, 1994) could result in a greater abundance of intact IGFBP-3 on Western ligand blot. However, analysis of proteolytic activity affecting IGFBP-3 in  $\text{TGF}\beta_1$  treated



Figure 7.vi.



The effect of TGF $\beta$  1 on IGFBP-3 protease activity produced by Hs68 fibroblasts. 24 hour conditioned medium (CM) from untreated or TGF $\beta$  1 (1 $\mu$ g/l) treated cells was incubated for 4 hours at 37 °C with  $^{125}\text{I}$ -IGFBP-3 and then subjected to electrophoresis. Lane 1 shows control IGFBP-3, lane 2 shows control protease (term pregnancy serum, PS), lane 3 shows PS + EDTA (25 mM), lanes 4 and 5 show untreated cell CM, lanes 6 and 7 show TGF $\beta$  1 treated cell CM, lanes 8 and 9 show untreated cell CM + EDTA (25 mM) and lanes 10 and 11 show TGF $\beta$  1 treated cell CM + EDTA.



Hs68 cell conditioned media failed to record any change compared to that taken from untreated controls. It appears therefore, that whilst we cannot discount a possible role for such a mechanism in regulating IGFBP-3 either associated with the cell surface or in more chronic  $\text{TGF}\beta_1$  treatments, it does not seem to be even partly responsible for our observed effect in 24 hour conditioned medium.

Another major function of  $\text{TGF}\beta_1$ , in parallel with its negative effect on proteases, is to enhance the synthesis of extracellular matrix components, and some functional aspects of  $\text{TGF}\beta_1$  have been ascribed to such secondary effects. The ability of  $\text{TGF}\beta_1$  to stimulate growth of normal cells in semi-solid media is accompanied by enhanced fibronectin secretion and can be inhibited by the addition of synthetic peptides which block subsequent interaction of the fibronectin with cell surface integrin receptors. The interesting aspect from an IGFBP standpoint is that the integrin receptor binding domains act through recognition of RGD (Arg-Gly-Asp) amino acid sequences, found repeatedly throughout the fibronectin molecule. As I have described in the Introduction, a single such sequence can also be found in both IGFBP-1 and IGFBP-2, thus raising the exciting possibility, albeit somewhat remote, of IGFBP-3 being regulated by other IGFBPs. Unfortunately, our experimental data found that exogenous fibronectin had no effect upon IGFBP-3 abundance in fibroblast conditioned medium, and the response to  $\text{TGF}\beta_1$  was not affected by co-incubation with the RGD sequence peptide. This would strongly suggest that the integrin receptor / fibronectin is not a novel pathway of IGFBP-3 regulation and the functional significance, if any, of the RGD sequence in IGFBP-1 and -2, still remains to be deduced.

In addition to its effect on IGFBP-3,  $\text{TGF}\beta_1$  also had an effect upon the appearance of IGFBP-4 and IGFBP-5 in Hs68 conditioned medium. By Western ligand blotting both of these binding proteins were reduced some 50 - 60 % in response to 1  $\mu\text{g/l}$   $\text{TGF}\beta_1$ . However, analysis of IGFBP-4 mRNA abundance suggested that the  $\text{TGF}\beta_1$  induced change in this IGFBP was not due to altered gene expression in contrast to the apparent mechanism involved in IGFBP-3 mediation. A similar post-transcriptional pattern of IGFBP-4 regulation has been observed in fetal skin fibroblasts following treatment with IGF-I (Camacho-Hubner *et al*, 1992) apparently involving the enhancement of a specific

protease. Although we cannot dismiss the possibility that TGF $\beta_1$  may act in a similar manner this may seem somewhat unlikely given the generally negative effect of TGF $\beta_1$  on proteolytic enzymes.

Alternatively, a reduction in IGFBP-4 levels in conditioned media may theoretically result from an increase in the proportion of cell-associated IGFBP-4. Although the latter would seem an attractive hypothesis in view of the stimulating effect of TGF $\beta_1$  on extracellular matrix production, a recent study by Jones *et al* (1993) failed to detect any IGFBP-4 in the matrix from fetal fibroblasts. Other possible mechanisms to explain the negative effect of TGF $\beta_1$  on IGFBP-4 peptide include altered mRNA translation or peptide secretion, although we have no data on these.



**CHAPTER 8. THE EFFECT OF ENDOGENOUS IGFBP**  
**REGULATION UPON IGF-I BIOACTIVITY**

## **OVERVIEW**

As we have seen in the previous chapters a number of growth factors and cytokines have the ability to regulate IGFBPs *in vitro*, via a number of mechanisms. Moreover, these effects can be elicited at peptide levels consistent with their physiological concentrations, suggesting they may have important consequences in controlling IGF bioactivity *in vivo*. The precise functions of the IGFBPs have not been defined however, although various effects of exogenously added IGFBPs have been reported in a number of *in vitro* IGF bioassays. Several forms of IGFBPs have been shown to have IGF binding affinity constants greater than those of the IGF receptors, resulting in the inhibition *in vitro* of IGF mitogenesis and metabolic processes such as glucose and amino acid transport. Conversely, enhancement of IGF actions by IGFBP-1 and IGFBP-3 has also been observed. Even though such opposing effects are often dependent upon the specific incubation conditions of the bioassay, they may reflect problems in reproducing the effects of endogenously secreted IGFBPs with purified or recombinant peptides.

The aim of this chapter was therefore to examine the consequences on IGF-I bioactivity of altered endogenous IGFBP secretion, induced in our Hs68 fibroblast cells by  $\text{TNF}\alpha$  and  $\text{TGF}\beta_1$ . This was undertaken by the development of a precise cytochemical bioassay, measuring IGF-I mitogenesis in these cells. To delineate the effects of the IGFBPs we compared native IGF-I with the IGF-I analogue with minimal IGFBP affinity, LongR3-IGF-I.

## **8.1. METHODS**

### **8.1.i. Cell culture**

Hs68 fibroblasts were plated into 96-well microtitre tissue culture plates at a density of  $3.75 \times 10^3$  / well ( $2.5 \times 10^4$  / ml) in a volume of 150  $\mu\text{l}$  DMEM + 10% fetal calf serum. After 96 hours at 37°C and 5%  $\text{CO}_2$ , the spent medium was aspirated and replaced with DMEM + 0.1 % BSA and incubated for a further 24 hours. Following aspiration of this medium, samples were added for 48 hours before measurement of cell number with the MTT assay, as described in Materials and Methods.



## **8.2. RESULTS**

### **8.2.i. Effects of IGF-I**

Addition of IGF-I to Hs68 human fibroblasts resulted in a demonstrable increase in cellular MTT reduction after 48 hour incubations. No effect was seen after 24 hours. Corresponding haemocytometer analysis of similarly treated fibroblasts grown in larger 6-well culture plates showed good correlation of cell number with the MTT response (**figure 8.i.**), although the latter bioassay was considerably more precise. Sensitivity of the cells to IGF-I was relatively low, with activity observed at doses above 10 µg/l and an effective range from 25 to 100 µg/l (3.3 - 13 nmol/l) (**Figure 8.ii.**). Maximal responses to IGF-I were typically observed in a number of similar experiments at between 30 - 40% above serum-free controls.

LongR3-IGF-I is an IGF-I analogue that consists of the complete human IGF-I sequence with a substitution of Arg for Glu at position 3, and a 13 amino acid extension at the N-terminus (Ballard *et al*, 1986). These structural alterations result in greatly reduced binding affinities for all IGFBPs, whilst minimally affecting its type I IGF receptor affinity. Consequently its ability to stimulate Hs68 fibroblast proliferation was considerably greater than that of the native peptide (**figure 8.ii.**). Significant activity was observed at LongR3-IGF-I doses above 0.1 µg/l (0.013 nmol/l), some 20 - 25 fold lower than IGF-I. In a number of experiments its effective dose range was between 0.25 and 10 µg/l (0.033 - 1.3 nmol/l).

### **8.2.ii. Exogenous IGFBP-3**

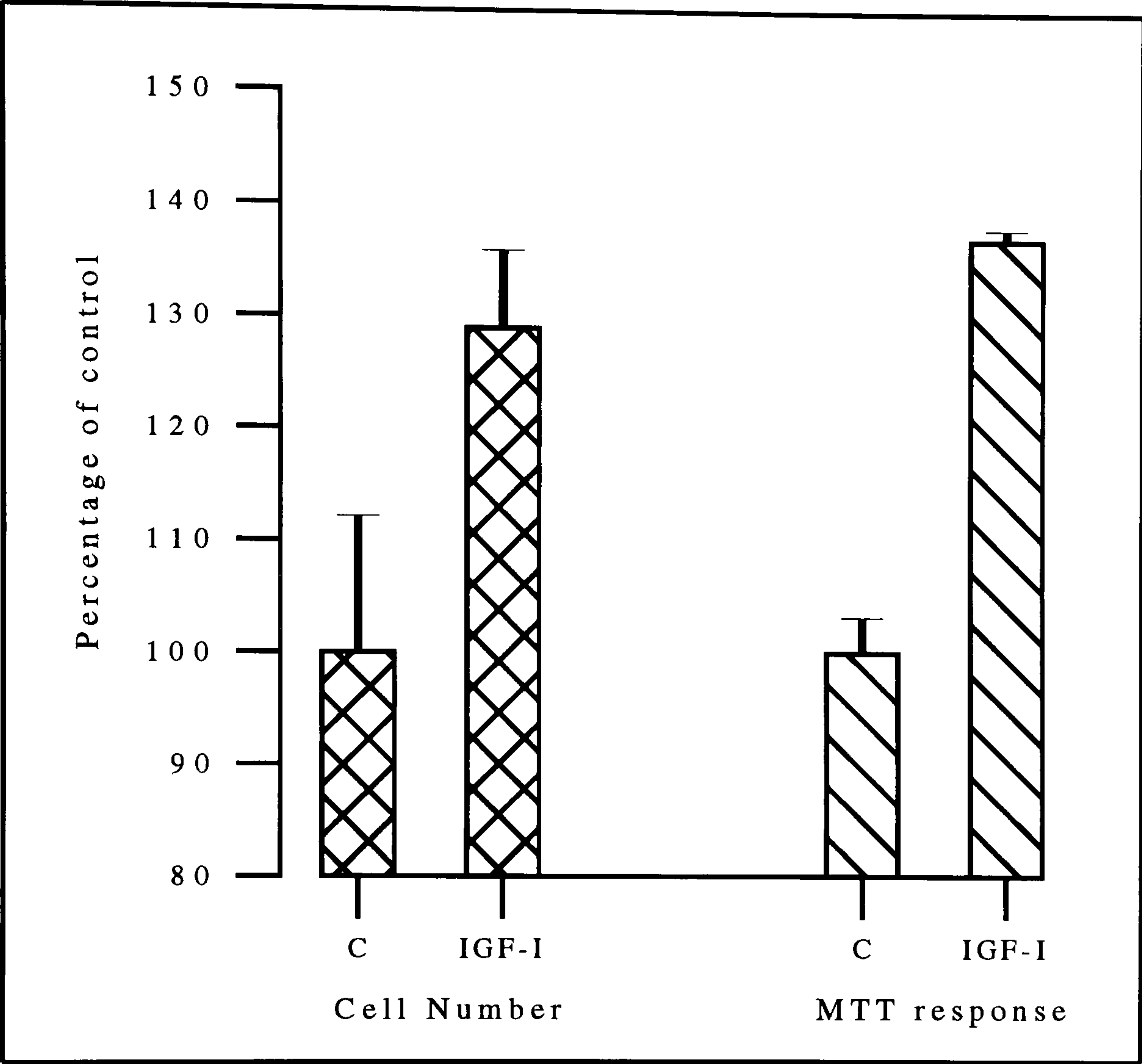
The effects of human recombinant IGFBP-3 (non-glycosylated) on the mitogenic activities of the two IGF-I peptides was investigated using the MTT assay.

#### **Co-incubation**

The inclusion of large doses of IGFBP-3 (5 mg/l) during the 48 hour cell incubation with IGF-I resulted in the complete inhibition of its mitogenic response up to doses of 50 µg/l (**figure.8.iii.a.**). Lower levels of IGFBP-3 were not as effective, indicating a non-equimolar relationship in this response.

The co-incubation of this high IGFBP-3 concentration with the IGF-I analogue, LongR3-

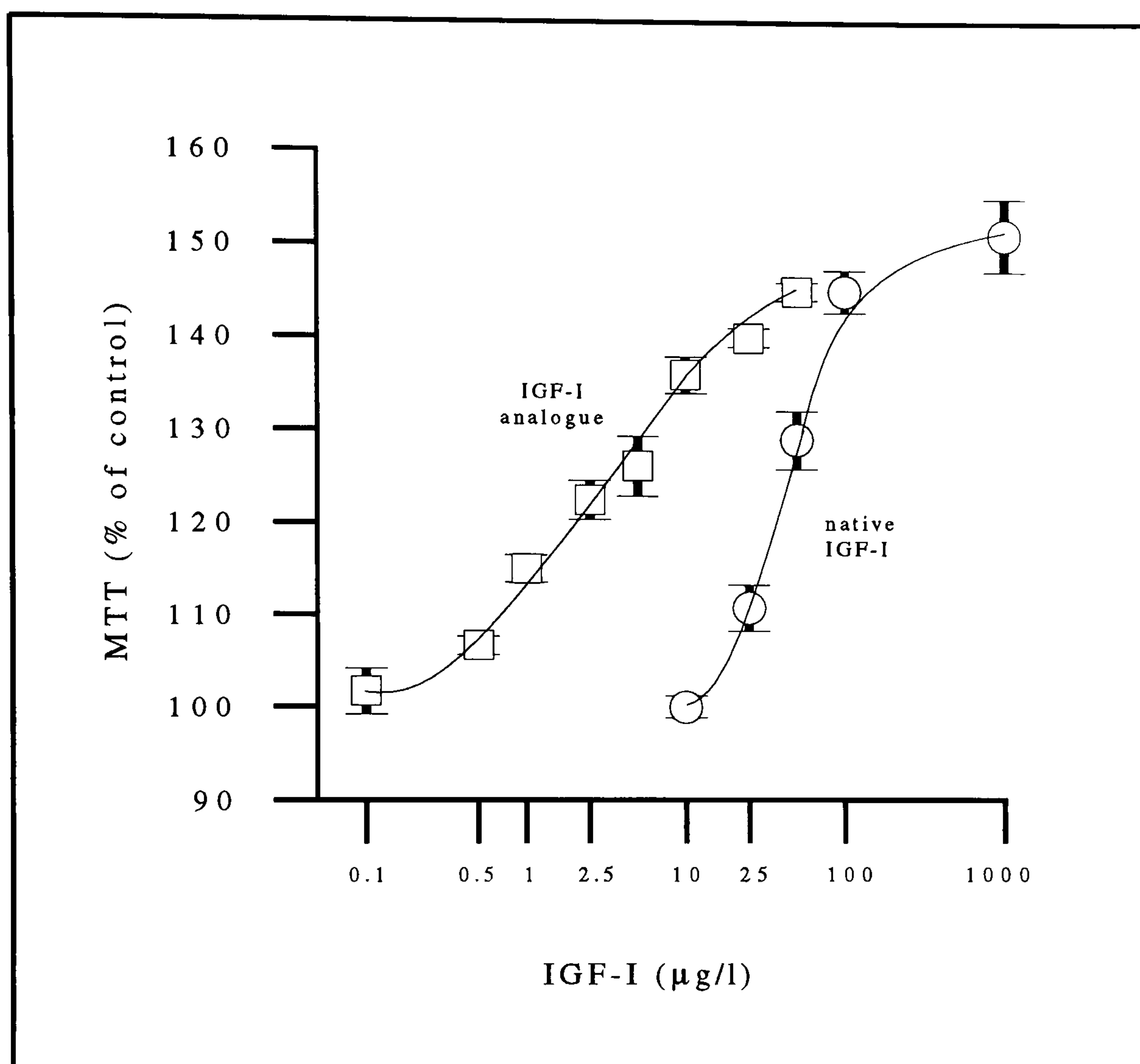
Figure 8.i.



Comparison of the MTT response and cell number following 48 hour stimulation of Hs68 fibroblasts with 100µg/l IGF-I. Cell number was assessed with a haemocytometer. Means from triplicate cultures  $\pm$  S.D. are expressed as the percentage cell count or MTT response compared to the serum-free control (C) values.

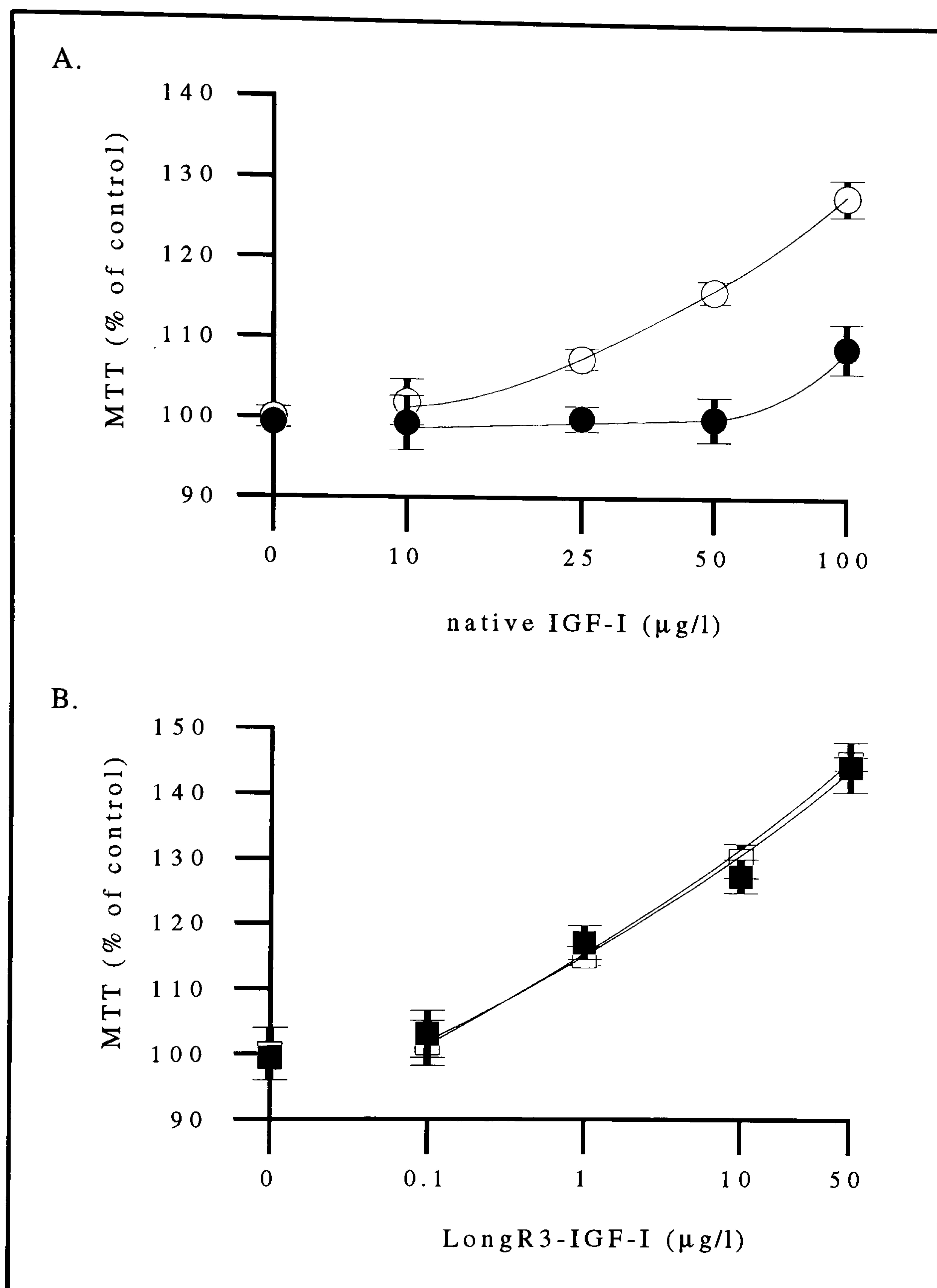


Figure 8.ii.



Dose-dependent stimulation of Hs68 cell mitogenesis by native IGF-I (○) and LongR3-IGF-I (□) assessed by the MTT assay. Cells were cultured in serum-free conditions for 48 hours with IGF-I before addition of MTT as described in the Methods. Results are means  $\pm$  S.D. of triplicate wells expressed as percentage optical density of serum-free controls.

Figure 8.iii.



The effect on Hs68 cell proliferation of co-incubating exogenous IGFBP-3 (5mg/l) with a dose-range of either native IGF-I or LongR3-IGF-I. Following incubations of 48 hours at 37°C, cultures were committed to the MTT assay as described in the Methods. Shown in (A) is native IGF-I alone (○) and IGF-I + IGFBP-3 (●), and in (B) LongR3-IGF-I alone (□) and LongR3-IGF-I + IGFBP-3 (■). Results are means of triplicate wells  $\pm$  S.D. expressed as the percentage MTT of serum-free controls.



IGF-I, was without effect on its mitogenic activity (**figure.8.iii.b.**) thus demonstrating its nominal affinity for the IGFBPs.

### **Pre-incubation**

The confusion that exists at present concerning the functional role of IGFBP-3 has largely arisen from a number of studies describing the ability of exogenous IGFBP-3 to enhance IGF activity *in vitro*. Both enhancement and inhibition of IGF-I can occur within the same cell line and appears to depend upon the prior incubation of IGFBP-3 with the cells before the addition of IGF-I. To clarify the function of IGFBPs by examining the effects of endogenous rather than exogenous IGFBP-3 it was therefore important that our particular cell line had the capacity to exhibit both these effects.

The effect of a 48 hour pre-incubation of exogenous IGFBP-3 on the mitogenic activity of 50 µg/l IGF-I in Hs68 fibroblasts is shown in **figure 8.iv**. Enhancement of IGF-I activity was observed and was dose-dependent with a maximal increase of 25.3 % ± 5.4 at 100 µg/l IGFBP-3, largely in agreement with Baxter *et al* (1988). No further effects were seen at higher doses of IGFBP-3.

Interestingly, IGFBP-3 pre-incubation also enhanced the mitogenic action of LongR3-IGF-I in Hs68 fibroblasts. Maximal increases of 18.6 % ± 1.5 (**figure 8.iv.**) were again evident at 100 µg/l IGFBP-3 with reduced values at higher doses thereafter.

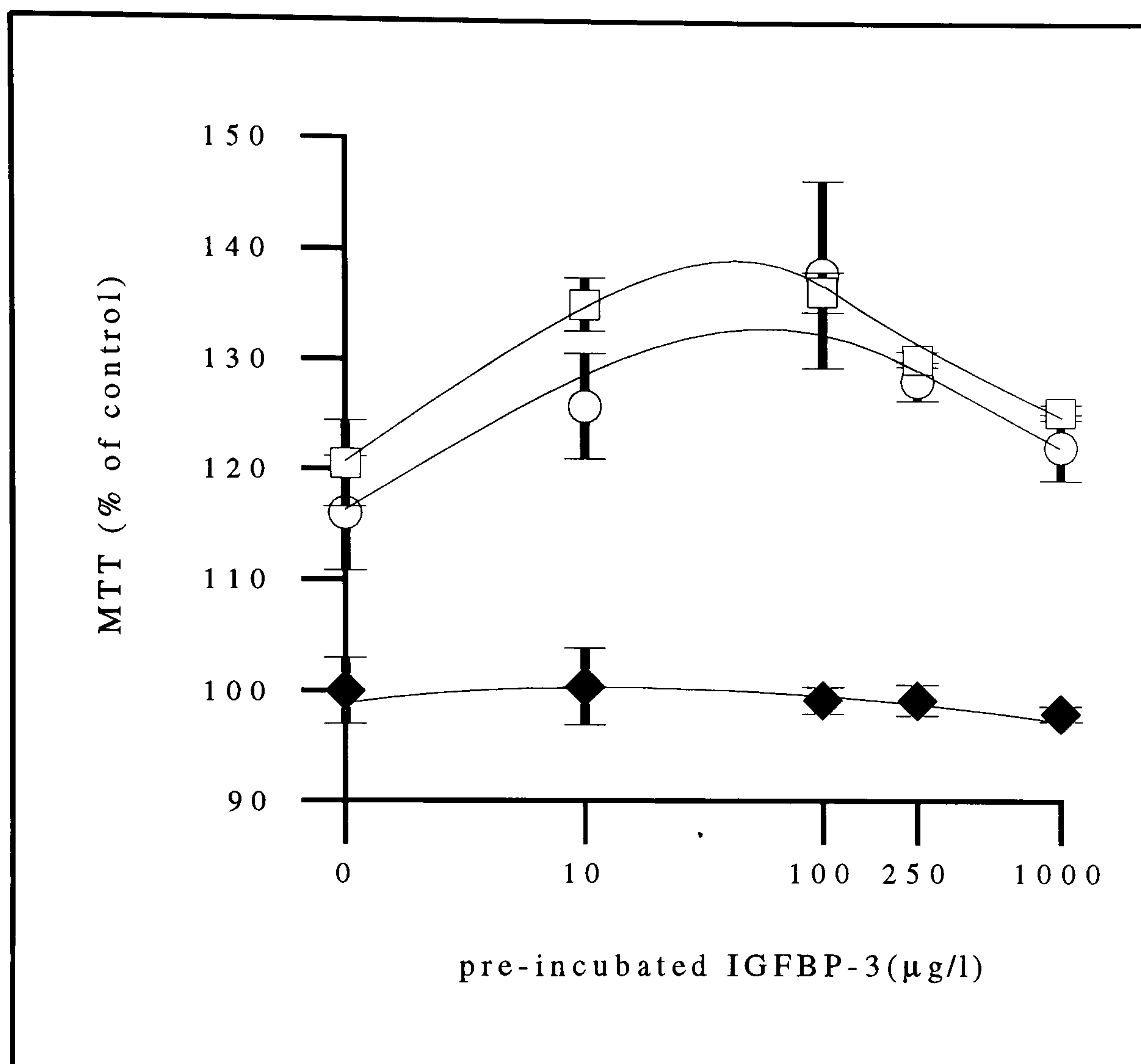
### **8.2.iii. Endogenous IGFBP regulation**

Satisfied that exogenous IGFBP-3 could both inhibit and enhance IGF-I in our Hs68 fibroblast cell line, we then investigated the effects on IGF-I mitogenesis of endogenous IGFBP-3 regulation by TNFα and TGFβ<sub>1</sub>.

#### **TNFα**

As described in Chapter 5 of this thesis the inflammatory cytokine TNFα inhibits the presence of Hs68 fibroblast IGFBPs in serum-free conditioned medium. However as this factor can be cytotoxic in a number of cell types it was important that its direct growth regulatory effects were investigated. When assessed using the MTT cytochemical assay, TNFα caused a dose-dependent increase in cell proliferation after 48 hour incubations, with maximum increases of between 50 - 60% above the control (**figure 8.v.**). No effect

Figure 8.iv.

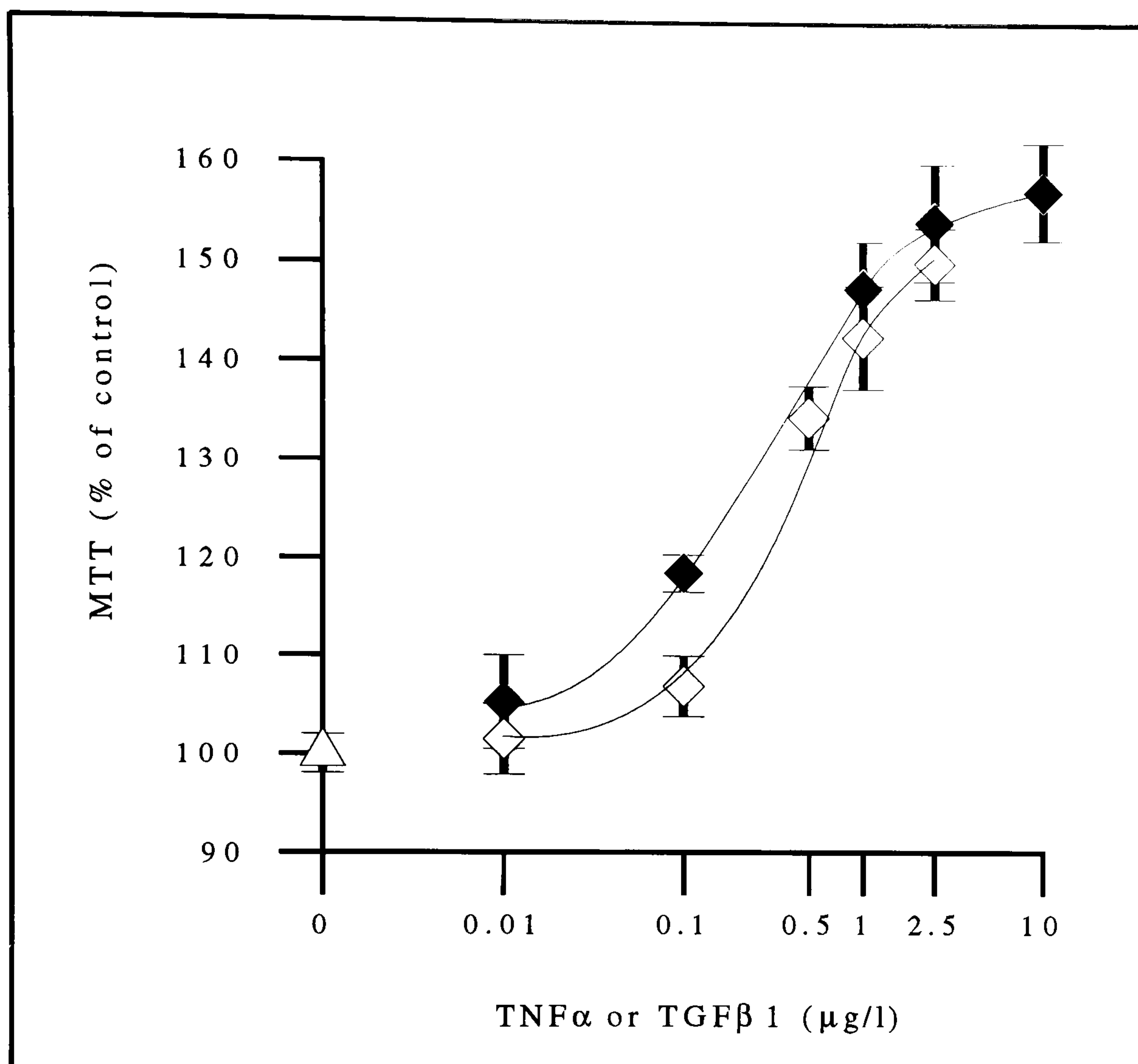


The effect of pre-incubating a dose-range of exogenous IGFBP-3 on the mitogenic activity of native IGF-I and LongR3-IGF-I in Hs68 cells. IGFBP-3 was incubated with the cells for 48 hours, aspirated and replaced for a further 48 hours with either serum-free media (♦), 50 μg/l native IGF-I (○) or 5 μg/l LongR3-IGF-I (□). Cell number was then assessed by the MTT assay. Results are means of triplicate wells  $\pm$  S.D. expressed as the percentage MTT of the serum-free controls.





Figure 8.v.



Dose-dependent stimulation of Hs68 cell proliferation by tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) and transforming growth factor  $\beta$  1 (TGF $\beta$  1), assessed by the MTT assay. Cells were cultured in serum-free conditions for 48 hours with either TNF $\alpha$  (◆) or TGF $\beta$  1 (◇) before the addition of MTT as described in the Methods. Results are means  $\pm$  S.D. of triplicate wells expressed as the percentage optical density of serum-free controls.

was observed after 24 hours. Its effective range was between 0.01 - 10  $\mu\text{g/l}$  (0.6 - 600 pmol/l), some one hundred-fold less than the native IGF-I. This would strongly suggest therefore that the apparent reduction in IGFBP secretion over 24 hours by  $\text{TNF}\alpha$  was not due to cytotoxicity.

The  $\text{TNF}\alpha$  dose (1  $\mu\text{g/l}$ ) seen to maximally decrease IGFBP-3 secretion was subsequently co-incubated with a dose range of IGF-I and cell proliferation assessed by the MTT bioassay. As seen in **figure 8.vi.**, fibroblast sensitivity to IGF-I was increased approximately five-fold, with responses above that of  $\text{TNF}\alpha$  alone observed at IGF-I concentrations of 10  $\mu\text{g/l}$ . Interestingly however, the response to the highest IGF-I dose examined, 100  $\mu\text{g/l}$ , was significantly ( $p < 0.05$ ) reduced in the presence of  $\text{TNF}\alpha$ .

Similar experiments were then performed with a range of LongR3-IGF-I concentrations co-incubated with 1  $\mu\text{g/l}$   $\text{TNF}\alpha$  to assess the role of IGFBPs in the above response. No such increase in fibroblast sensitivity to the analogue by  $\text{TNF}\alpha$  was seen (**figure 8.vii.**). Indeed, although sensitivity remained unaltered in the presence of  $\text{TNF}\alpha$ , the degree of response to LongR3-IGF-I was significantly reduced ( $p < 0.05$ ), perhaps indicating some form of IGF-I resistance.

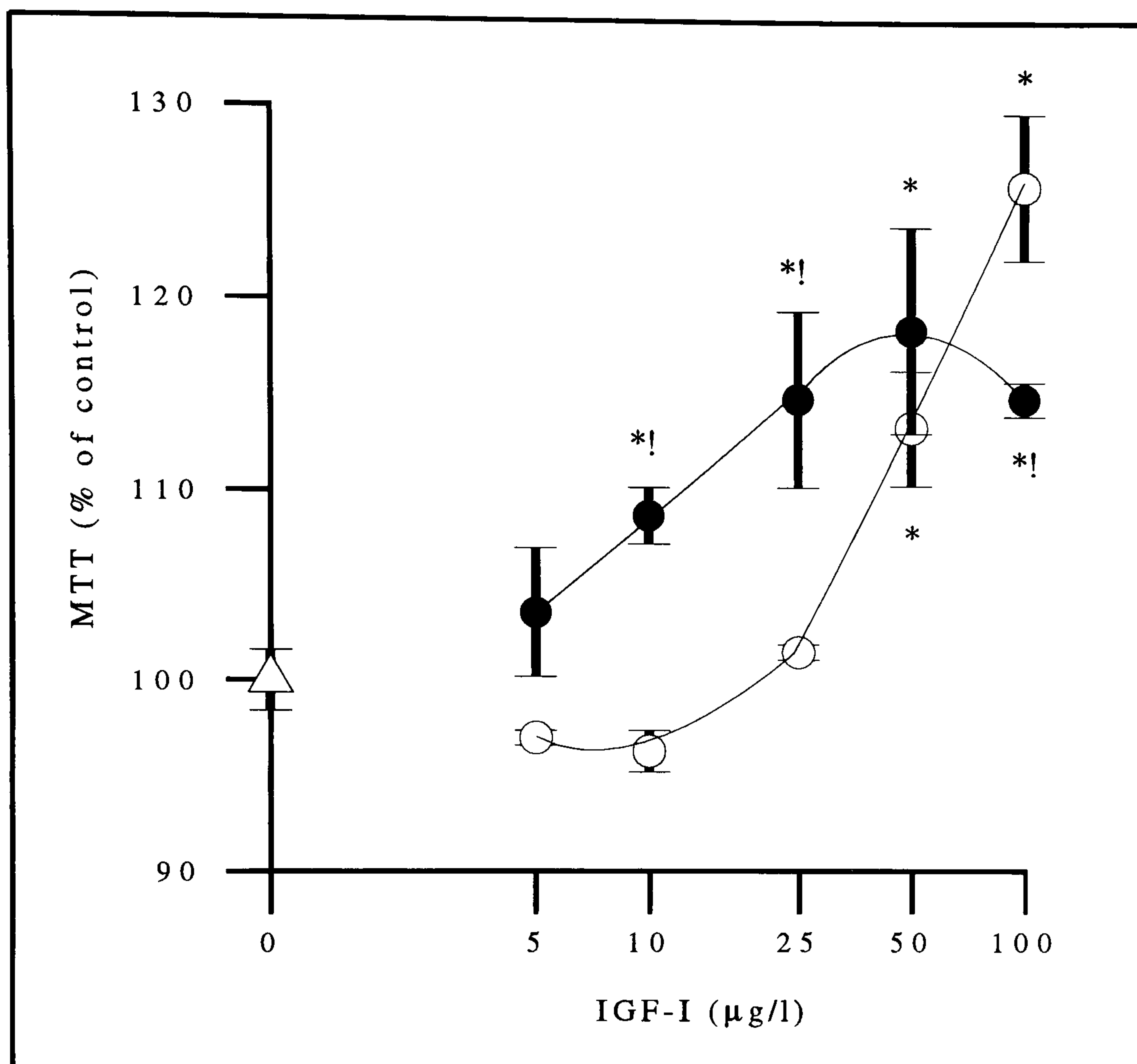
### **TGF $\beta_1$**

As described in Chapter 7 of this thesis, TGF $\beta_1$  is a potent stimulator of Hs68 fibroblast IGFBP-3 secretion, with its effect mediated via enhanced gene expression. As such, it would seem to provide a good model to investigate the effects of increased endogenous IGFBP-3 secretion upon IGF-I bioactivity. Moreover, as the secretion of other IGFBPs produced by this cell line (IGFBP-4 and IGFBP-5) appeared to be reduced in response to this factor, in a similar manner to  $\text{TNF}\alpha$ , it could be argued that any divergence in the effect of the two peptides on IGF activity is likely to be due to IGFBP-3.

TGF $\beta_1$ , in the absence of serum, was found to cause a dose-dependent increase in MTT response over a 48 hour incubation period (**figure 8.v.**). In a similar fashion to the response observed to  $\text{TNF}\alpha$ , no effect was seen after 24 hours, the incubation period used for analysis of IGFBP modulation. Typically, maximal responses reached 50 - 60% above control values, and its effective concentration ranged from 0.05 - 10  $\mu\text{g/l}$  (2 - 400 pmol/l).



Figure 8.vi.

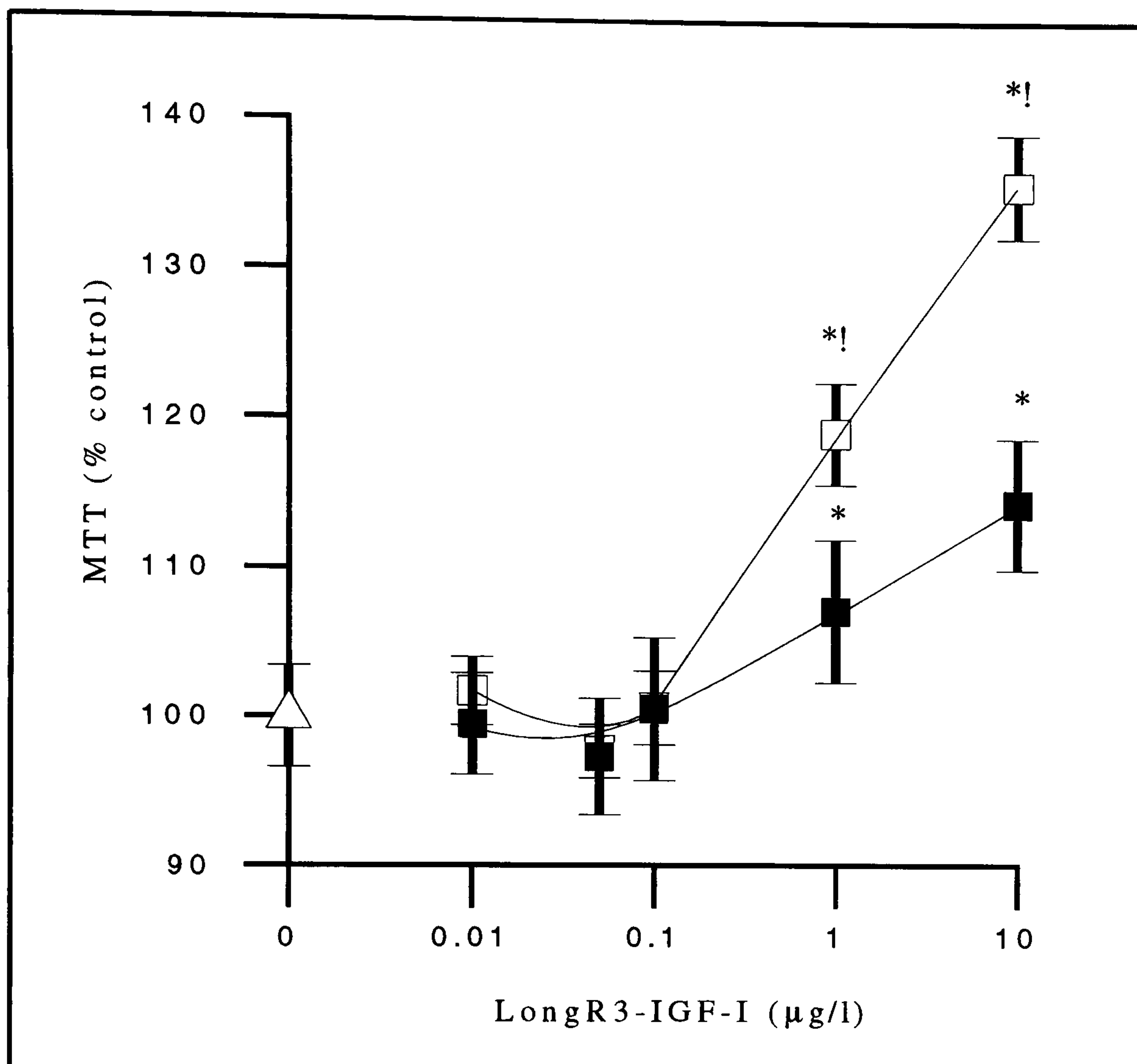


The effect on Hs68 cell proliferation of co-incubating native IGF-I with tumour necrosis factor  $\alpha$  (TNF $\alpha$ ). Cells were incubated with a dose-range of IGF-I in the presence or absence of 1  $\mu$ g/l TNF $\alpha$  for 48 hours before the addition of MTT, as described in the Methods. The results are expressed as the percentage optical density of either serum-free controls for IGF-I alone (○), or of TNF $\alpha$  for IGF-I + TNF $\alpha$  (●). Each point is the mean of triplicate wells  $\pm$  S.D.

\* P < 0.05 compared with control;

! P < 0.05 compared with the same dose of IGF-I in the absence of TNF $\alpha$ .

Figure 8.vii.



The effect on Hs68 cell proliferation of co-incubating LongR3-IGF-I with tumour necrosis factor  $\alpha$  (TNF $\alpha$ ). Cells were incubated with a dose-range of LongR3-IGF-I in the presence or absence of 1  $\mu$ g/l TNF $\alpha$  for 48 hours before the addition of MTT, as described in the Methods. Results are expressed as the percentage optical density of serum-free controls for LongR3-IGF-I alone ( $\square$ ), or of TNF $\alpha$  for LongR3-IGF-I + TNF $\alpha$  ( $\blacksquare$ ). Each point is the mean of triplicate wells  $\pm$  S.D.

\*  $P < 0.05$  compared with control;  
!  $P < 0.05$  compared with the same dose of LongR3-IGF-I in the presence of TNF $\alpha$ .



A single dose of TGF $\beta_1$  (1  $\mu$ g/l), seen to maximally stimulate IGFBP-3 secretion in Hs68 fibroblasts was then co-incubated with the effective dose range of IGF-I. Assessment of mitogenic activity over 48 hours was again made using the MTT assay. As can be seen in **figure 8.viii.**, IGF-I bioactivity above that of 1  $\mu$ g/l TGF $\beta_1$  alone was inhibited, with a significant response ( $P < 0.05$ ) observed only at the highest IGF-I dose tested, 100  $\mu$ g/l. This represented an apparent four-fold loss in sensitivity to the IGF peptide, and suggested that the increase in endogenous IGFBP-3 in response to TGF $\beta_1$  was inhibitory.

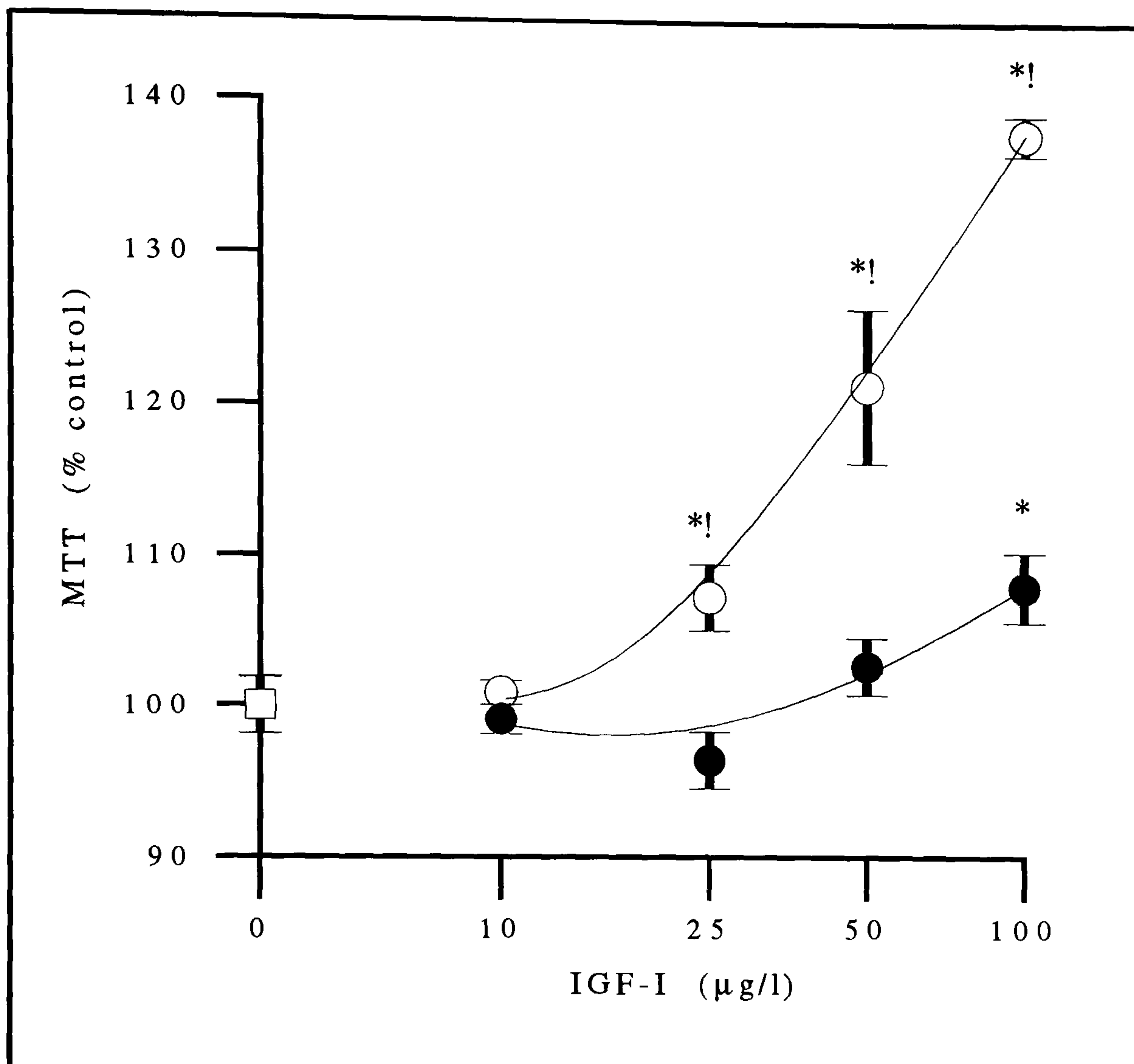
To confirm the involvement of the IGFBPs in this effect similar experiments were conducted, but with the replacement of the native IGF-I with a dose-range of the LongR3-IGF-I analogue. Due to the negligible affinity for the IGFBPs, those effects on IGF-I bioactivity mediated through the IGFBPs by TGF $\beta_1$  should be absent. Indeed, co-incubation of TGF $\beta_1$  (1  $\mu$ g/l) with LongR3-IGF-I was seen to be without consequence on the mitogenic activity the IGF-I analogue (**figure 8.ix.**) with no alteration in cell sensitivity or absolute response. This would strongly suggest that the inhibitory effect of TGF $\beta_1$  on native IGF-I was mediated via alteration in IGFBPs.

### **8.3. CONCLUSIONS**

#### **IGF-I activity**

This chapter has demonstrated that certain factors capable of regulating endogenous IGFBP secretion, at least *in vitro*, can elicit marked effects upon IGF-I bioactivity. The most frequently used bioassay to measure the mitogenic activity of the IGFs has been the cellular uptake and incorporation of radiolabelled [ $^3$ H]-thymidine. However, these assays have a number of disadvantages (see Introduction) and were not used to assess IGF activity in this study. Instead, we have developed a precise serum-free cytochemical bioassay, based upon that by Ealey *et al* (1988), capable of analysing numerous low volume samples within a single microtitre. The assay utilised the Hs68 fibroblast cell line, as used for the IGFBP secretion experiments, and did not require the use of isotopes or time-consuming extraction techniques. Briefly, the assay was able to measure cell proliferation, as the reduction by cellular dehydrogenase enzymes of the yellow tetrazolium MTT to its purple formazan was, in this particular model, a function of cell number. Moreover, cell counts of *stimulated* cells positively correlated with reduction of

Figure 8.viii.



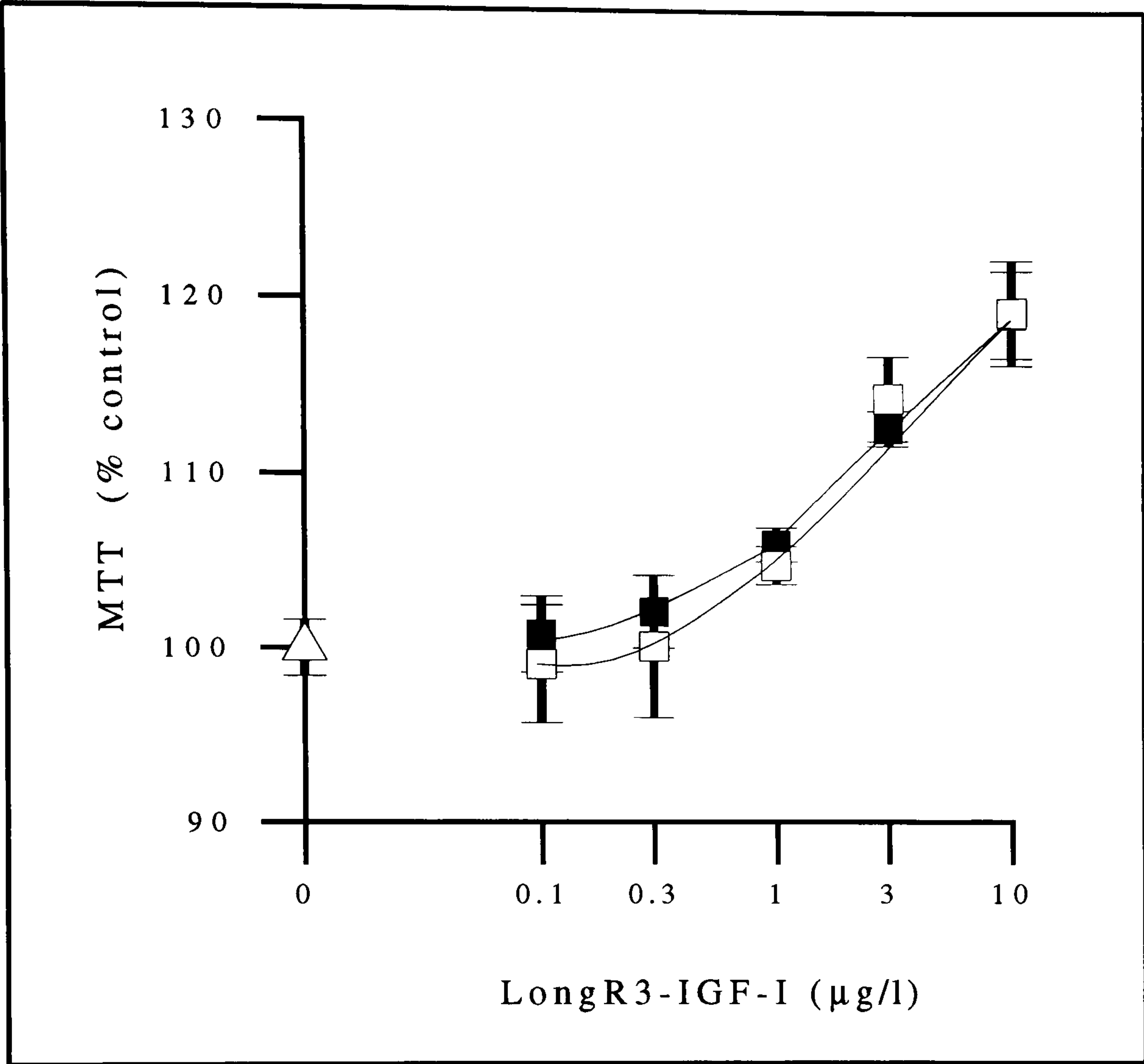
The effect on Hs68 cell proliferation of co-incubating native IGF-I with transforming growth factor  $\beta_1$  (TGF $\beta_1$ ). Cells were incubated with a dose-range of IGF-I in the presence or absence of 1  $\mu$ g/l TGF $\beta_1$  for 48 hours before the addition of MTT, as described in the Methods. The results are expressed as the percentage optical density of either serum-free controls for IGF-I alone (○), or of TGF $\beta_1$  for IGF-I + TGF $\beta_1$  (●). Each point is the mean of triplicate wells  $\pm$  S.D.

\*  $P < 0.05$  compared with control;

!  $P < 0.05$  compared with the same dose of IGF-I in the presence of TGF $\beta_1$ .



Figure 8.ix.



The effect on Hs68 cell proliferation of co-incubating LongR3-IGF-I with transforming growth factor  $\beta_1$  (TGF $\beta_1$ ). Cells were incubated with a dose-range of LongR3-IGF-I in the presence or absence of 1  $\mu$ g/l TGF $\beta_1$  for 48 hours before the addition of MTT, as described in the Methods. Results are expressed as the percentage optical density of serum-free controls for LongR3-IGF-I alone ( $\square$ ), or of TGF $\beta_1$  for LongR3-IGF-I + TGF $\beta_1$  ( $\blacksquare$ ). Each point is the mean of triplicate wells  $\pm$  S.D.

MTT, in a similar fashion to that seen in an IGF bioassay recently published using BALB/c 3T3 fibroblasts (Okajima *et al.*1992).

Despite numerous data on the need for certain 'competence factors' to render cells sensitive to the mitogenic actions of IGF-I, there was no such requirement in our Hs68 cells using the MTT assay. Dose-dependent IGF-I mediated increases in cell number were observed in serum-free medium after 48 hour incubations. This however, is not without precedent (Campisi and Pardee,1984) and has been observed in exponentially growing cells when they have not entered into quiescence or  $G_0$  of the cell cycle. Presumably, the 24 hour serum-free pre-incubation that our cells receive before the addition of the IGF-I is not sufficient to take them into  $G_0$ . Thus, cells in the  $G_1$  resting phase of the cell cycle can be induced by IGF-I to progress into the  $S$  phase, resulting in increased cell number.

### **Modulating effects of IGFBPs**

IGF-I sensitivity in our human Hs68 cells was relatively low, with effective doses only observed above 10  $\mu\text{g/l}$ , compared with 0.5  $\mu\text{g/l}$  seen in the BALB/c 3T3 assay (Okajima *et al.*, 1992). As these mouse cells were reported to produce little or no IGFBPs, an attractive hypothesis would be that the endogenous IGFBPs produced by our Hs68 cells were acting in an inhibitory manner. This function of the binding proteins was subsequently demonstrated by the activity of an IGF-I analogue with negligible affinity for all IGFBPs, LongR3-IGF-I, which was twenty-fold more potent in the MTT assay. This would appear to be in agreement with a number of previous studies using fibroblast cell culture models that have shown that purified and recombinant IGFBP-3, when co-incubated with IGF-I, inhibits subsequent IGF actions (DeMellow and Baxter,1988; Conover,1991). However, these same IGFBP-3 preparations can also enhance IGF-I when they are pre-incubated with the cells before the addition of the IGF, thus casting doubt onto its precise function. As both of these apparently opposing effects were observed in our fibroblast cell line, it was felt these cells represented a valid model to investigate the effect on IGF-I activity of alterations in *endogenous* IGFBP secretion, specifically by  $\text{TNF}\alpha$  and  $\text{TGF}\beta_1$ . Delineation of those effects on IGF-I bioactivity mediated via alteration in IGFBP secretion was achieved by comparing native IGF-I and LongR3-IGF-I, the



analogue with little affinity for the IGFBPs.

We have subsequently observed that TNF $\alpha$  enhanced IGF-I bioactivity in Hs68 fibroblasts and that this appeared to be due to its ability to inhibit the secretion of IGFBPs produced by these cells. Specifically, this cytokine lead to an approximate five-fold increase in sensitivity to native IGF-I, a consequence presumably created by greater access of the IGF-I peptide to cell receptors. Confirmation of IGFBP involvement was provided by data showing no such increase in sensitivity to the LongR3-IGF-I analogue. The lack of a TNF $\alpha$ -induced enhancement of 100 $\mu$ g/l IGF-I may perhaps be explained by the IGF receptors being fully saturated at this maximally effective dose even in the absence of TNF $\alpha$ , and is thus consistent with a central role for IGFBP modulation in the sensitizing effect.

In addition to TNF $\alpha$  affecting IGF-I bioactivity via IGFBP regulation, a more complex relationship appears to exist however. In the presence of TNF $\alpha$ , mitogenic responses to LongR3-IGF-I (1 - 10  $\mu$ g/l) and the highest dose of the native peptide (100  $\mu$ g/l) were reduced, perhaps indicating some form of receptor-mediated IGF-I resistance. There is some indirect evidence that TNF $\alpha$  may induce IGF-I receptor resistance in rat muscle and skin (Lang *et al*, 1992) with hyperinsulinaemia failing to increase glucose uptake to that seen in non-TNF $\alpha$  infused animals. In this *in vivo* model, it was proposed that due to cross-reactivity of such high insulin levels at least some of its activity would be directed through the IGF-I receptors. Presumably, in our Hs68 fibroblasts TNF $\alpha$  enhancement of low levels of native IGF-I results from increased access of IGF-I to its receptors following inhibition of IGFBP secretion outweighing the moderate attenuation of receptor function.

The TGF $\beta$  group of regulatory molecules are, like other cytokines and growth factors, multifunctional regulators of cellular activity. They can stimulate cell proliferation, growth and differentiation, but are also able to inhibit such processes, depending on cell type and incubation conditions. In contrast to the effect of TNF $\alpha$ , TGF $\beta_1$  was seen to inhibit IGF-I mitogenesis through its modulation of IGFBPs. This would appear to result from an increase in IGFBP-3 secretion, as other IGFBPs were reduced on ligand blot in a similar manner to TNF $\alpha$ . However, since a number of these 'minor' IGFBPs can be localized in the extracellular matrix (ECM), we could speculate that an increase in ECM in response

to TGF $\beta_1$  (Ignatz and Massague,1986) could effectively remove them from the conditioned medium. This would effectively provide additional inactive binding sites for localization of the IGF-I peptide, thus reducing its bioactivity further. Without this data we are left to conclude that the contrasting actions of TNF $\alpha$  and TGF $\beta_1$  on IGF-I bioactivity seem to result from their discordant effects upon endogenous IGFBP-3 secretion, the function of which appears to be inhibitory.



**CHAPTER 9.**  
**DISCUSSION**

## **SECTION A. IGF-I regulation of fibroblast IGFBP-3**

### **9.1. A function of protease modulation?**

Factors that may regulate IGF activity are varied and complex, and may include alteration in IGF gene expression, peptide secretion and receptor modulation. In addition, the existence of at least six molecularly distinct IGFBPs, often secreted in a tissue-specific manner, has seemingly produced a further dimension in the control of the IGF peptide. IGFBPs have high affinity for the IGFs and thus can both greatly stabilise the IGFs in the circulation and also directly affect the ability of the IGF peptide to interact with its tissue receptors. Consequently it is easy to imagine a concept of the IGFBPs acting as rather inert inhibitors of IGF bioactivity, simply buffering the growth factor as and when required for homeostatic control of cellular growth. Such a simplistic hypothesis has its drawbacks however, as it fails to explain how the IGFs are liberated to target tissues from either the circulating IGFBPs or from local IGFBPs at the tissues themselves. Clearly, to accomodate such a mechanism requires the existence of a more fluid or dynamic IGFBP system than that of simple IGF inhibition.

It was Clemmons and co-workers (1983) who first suggested the possibility that proteases could provide an effective mechanism that may lead to transfer of the IGFs from the IGFBPs to tissues. They described experimentally, the ability of human serum proteases and heparin to disrupt the high molecular weight ternary IGF/IGFBP-3 complex, resulting in an increase in the free form of the growth factor, presumably due to a reduction in IGFBP affinity. Despite the attraction and widespread potential of enzymic control of IGFBP function, it was not until two papers concerning the appearance of serum IGFBPs in pregnancy were published seven years later that major interest was generated in the possibility of such a system having a physiological role. Both studies, by Hossenlopp *et al* (1990) and Giudice (1990) described the appearance of proteolytic activity in term pregnancy serum, which affected IGFBP-3 such that it was largely absent on ligand blots despite reported two-fold elevations in its immunoreactivity (Baxter and Martin, 1986). The Hossenlopp study revealed that the pregnancy protease could be inhibited experimentally by the addition of either the divalent metal cheleter EDTA, aprotinin or high concentrations of phenylmethylsulfonyl-fluoride, suggesting the presence of a serine



type enzyme. It was these studies therefore that appeared to provide the first evidence in a physiological situation of protease regulation of IGFBPs, thus supporting the original findings of Clemmons *et al* (1983).

Since this initial description of IGFBP-proteases, serum from a wide range of pathological conditions has been investigated in a similar manner, and it now appears that such activity is not restricted to pregnancy alone. Proteases principally affecting IGFBP-3 have now been described in catabolic patients suffering injury or post-operative stress, in various cancers (lung, breast, head and neck), poorly controlled diabetics, some subjects with severe GH hormone deficiency and in some GH-insensitivity syndrome (Laron) patients (Holly *et al*, 1993). In addition, IGFBP-protease activity has also been recorded in the conditioned medium from a number of cell types *in vitro*, including human breast cancer cells (Oh *et al*, 1993), rhabdomyosarcomas, cervical and bladder cancer cells, choriocarcinoma, and squamous cell carcinoma of the tongue (Holly *et al*, 1993). More detailed analysis has suggested the enzymes responsible for IGFBP degradation may include plasmin from osteosarcoma cells (Campbell *et al*, 1992), prostate-specific antigen (PSA) in seminal plasma (Cohen *et al*, 1992) and gelatinase-like enzymes in the circulation of non-human primates. Experimentally, a wide range of purified proteases also have the ability to degrade IGFBPs, such as collagenase, trypsin, cathepsin D, urokinase and plasminogen, leading to the suspicion that IGFBP proteolysis is widespread throughout the tissues and could be a common regulatory mechanism of IGF action *in vivo*. It would appear that the relatively large molecular size of the IGFBPs coupled with the functional importance of their complex folding pattern (most have at least 18 cysteine residues involved in forming disulphide bonds) may help explain why they seem somewhat vulnerable to proteolytic degradation.

In this thesis we have described the presence of IGFBP-3 protease in 24-hour serum-free conditioned media taken from a normal human dermal fibroblast cell line, Hs68, which in addition to that observed in numerous transformed tissues outlined above, further suggests the broad nature of this mechanism. Its activity, which could be inhibited by EDTA, was evident following sodium dodecyl sulphate (SDS) gel electrophoresis by



increased fragmentation of a  $^{125}\text{I}$  radiolabelled recombinant non-glycosylated form of the binding protein. Moreover, as this technique does not rely upon serum as a source of IGFBP-3, we can be assured that the activity originated from the fibroblasts and was not due to activation of serum proteases.

Detection of IGFBP protease has often relied upon observing discrepancies between IGFBP immunoassay data and ligand blot analyses. As the latter technique provides a measure of  $^{125}\text{I}$ -IGF-I binding affinity of the IGFBP the presence of protease is signalled by abnormally low values compared with immunoassay. In our fibroblast model the opposite was observed, with apparent IGF-I stimulation of IGFBP-3 seen on ligand blot only. As this effect was non-receptor mediated, suggesting the involvement of post-transcriptional processes such as protease modulation, we proposed that IGF-I was having a negative effect upon protease activity. Subsequently, IGF-I mediated protection of the IGFBP peptide from proteolytic degradation was observed, perhaps indicating the existence of a simple negative feedback system whereby increasing concentrations of IGF peptide lead to more effective inhibitory control by the IGFBPs. Similar IGF-I diminution of IGFBP protease activity, as opposed to inhibition of secretion, has now also been described in cultured human fibroblast cells for IGFBP-5 (Camacho-Hubner *et al*, 1992) and human breast cancer cells for IGFBP-3 (Oh *et al*, 1993).

The physiological role of IGFBP-proteases, which are not necessarily specific to the IGFBPs, is somewhat controversial in view of a number of data described by Baxter and colleagues. Following neutral size exclusion chromatography of human serum the molecular weight distribution of the IGFBP-3 peptide was found to be identical in pregnancy and non-pregnancy sera, with approximately 90% in the 150 kDa ternary complex (Suikkari and Baxter, 1992). Since the formation of this complex cannot occur unless IGFBP-3 binds IGF and  $\alpha$ -subunit normally, it was concluded that the IGFBP-3, supposedly proteolytically modified in the pregnancy serum, was functionally normal. The normal to elevated IGF levels seen in pregnancy serum (Hall *et al*, 1984) also suggested that the IGFBP-3 retained its capacity to bind IGF. It was argued that perhaps partial proteolytic hydrolysis lead to a reduction in the stability of the IGFBP, revealed only during the harsh biochemical treatment (sodium dodecyl sulphate and high temperatures)



used in the ligand blot electrophoresis. The necessity in the ligand blot technique for IGFBPs to be correctly refolded after their transfer onto nitrocellulose for them to recognise  $^{125}\text{I}$ -IGF (Binoux *et al*, 1991) provides indirect support for such a hypothesis. Questions concerning the validity of IGFBP-protease analysis were also raised by experiments that showed IGFBP-3 in pregnancy serum bound native IGF-I quite normally, but failed to bind the  $^{125}\text{I}$ -IGF-I used in ligand blotting (Suikkari and Baxter, 1991).

Although our assay for analysing putative IGFBP-proteases does not rely upon the use of serum or assessment of the binding capacity of IGFBP-3, it still relies upon SDS polyacrylamide gel electrophoresis. However, we have also shown that fragmentation of the recombinant IGFBP-3 peptide (albeit of dissimilar size to that evident after electrophoresis) can occur following incubation with fibroblast conditioned medium and separation under neutral conditions by size exclusion column chromatography. IGFBP-3 fragments have also now been recorded by sophisticated antisera in human serum samples (Blum 1993, *personal communication*), suggesting that such a process may reflect a physiological mechanism.

In conclusion, the involvement of proteolytic enzymes in regulating IGF release from inhibitory IGFBPs is an attractive concept, particular as it may provide us with an insight into the accelerated growth of transformed tissues. It is well recognised for example, that an important factor in tumour development and angiogenesis is the increased secretion of a wide variety of proteolytic enzymes. From our simple working hypothesis, we could speculate that high local protease concentrations may in effect activate IGFs, further contributing to tumour proliferation. However, the functional significance of these enzymes is still largely speculative and demands much greater clarification, not least because their proposed role is based upon the premise that IGFBPs are inhibitory to IGF action, which is not always the case.



## **SECTION B. Cytokine regulation of IGFBPs**

### **9.2. The relationship of TNF $\alpha$ and IL-1 to the IGFs**

With the great interest that now surrounds the existence of possible IGFBP proteases, we have investigated the effect on IGFBPs of a major group of signalling peptides, collectively known as cytokines, whose pleiotrophy of functions include the regulation of established cell derived proteases and inhibitors. Even without consideration of their effects upon proteases the cytokines may be likely candidates to be IGFBP regulators as they are often intrinsically involved with alterations in cellular growth and metabolism, either directly or through mediation of secondary factors.

In Chapter 2 of this thesis we have demonstrated that the major inflammatory cytokine tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) is indeed capable of regulating the secretion of IGFBP-3, IGFBP-4 and IGFBP-5 from the normal human dermal fibroblast cell line Hs68. By Western ligand blotting TNF $\alpha$  appeared to be inhibitory to all these IGFBPs, and a dose-dependent effect on IGFBP-3 was subsequently confirmed by radioimmunoassay. The effective TNF $\alpha$  doses of 0.25  $\mu$ g/l and above appear to be physiologically relevant, being consistent with those found pathologically in the plasma of sepsis patients (Casey *et al*, 1993), and with those that have been observed in the synovial fluid of arthritic joints infiltrated with activated macrophages and monocytes (Saez-Llorens *et al*, 1990).

Although the immunoassay data strongly suggested that TNF $\alpha$  was inhibiting the molar concentration of IGFBP-3 by altering its secretion, rather than enhancing protease activity, investigation of IGFBP-3 (and IGFBP-4) gene expression revealed that there was no parallel reduction in mRNA abundance during the 24 hour incubation period. This therefore consequently suggested the existence of some form of post-transcriptional mechanism in this effect.

In agreement with the immunoassay results, we could find no direct evidence to implicate TNF $\alpha$ -induction of IGFBP proteases in conditioned medium. However, it now appears that IGFBP proteolysis can be restricted to the cell surface, as demonstrated by the activation of cell-bound plasminogen by cell surface urokinase plasminogen activator (uPA) in osteosarcoma cells (Campbell *et al*, 1993). Although in our study there was a reduction in cell-bound IGFBPs following the TNF $\alpha$  treatment, perhaps resulting from enhancement



of such cell membrane proteases, this could equally result from down-regulated IGFBP secretion. Further studies investigating the role of TNF $\alpha$  in mediating IGFBP gene translation and / or secretory mechanisms will therefore be required to fully clarify the inhibitory effect of TNF $\alpha$ .

In addition to TNF $\alpha$ , the functionally closely related inflammatory cytokines IL-1 $\alpha$  and IL-1 $\beta$  were also capable of inhibiting IGFBP secretion from our normal fibroblast cells. Analysis of IGFBP-3 gene expression showed however, that IL-1 $\beta$  lead to a reduced abundance of this mRNA at the end of the 24 hour incubation period, in contrast to TNF $\alpha$ . This was unexpected in view of the many data reporting common second messenger systems and regulatory mechanisms employed by the two cytokines, such as activation of novel serine / threonine protein kinases and prostaglandin synthesis modulation (as reviewed in the Introduction).

It was found that exogenous prostaglandin E2 (PGE2) could lead to similar inhibition of Hs68 cell IGFBP-3, but the addition of a cyclo-oxygenase inhibitor, indomethacin, which blocks its synthesis, had no impact upon the TNF $\alpha$  effect. This may be a reflection of the high effective doses of PGE2 (some 1 mg/l) which simply may not be achieved from endogenous fibroblast secretion during the 24 hour TNF $\alpha$  incubation.

The differential effect of TNF $\alpha$  and IL-1 $\beta$  observed in our model at the molecular level is not without precedent however. For example, it has been demonstrated that protein synthesis is only required for TNF $\alpha$ , and not IL-1 $\beta$ , mediated up-regulation of low density lipoprotein receptor gene transcription in the human hepatoma cell line HEPG<sub>2</sub>, suggesting the activation of distinct signal transduction pathways (Stopeck *et al*, 1993). Additionally, the induction of IL-1 $\beta$  in human fibroblasts has been observed by Elias *et al* (1989) to be controlled at the gene level by IL-1 itself but involves post-transcriptional mechanisms for TNF $\alpha$ .

Notwithstanding the apparent divergence in the mechanisms used by TNF $\alpha$  and IL-1 $\beta$  to inhibit fibroblasts IGFBP-3 secretion, their effects may have quite wide ranging significance on IGF action *in vivo*. However, to interpret the role that such IGFBP modulation may have in regulating the IGFs we have to consider both their function as a buffer or reservoir for the IGF peptide, thus greatly increasing its stability, and also their



direct regulatory effects upon IGF / IGF receptor interaction at the tissue level. The latter point has been addressed by the development of a precise IGF bioassay in this thesis using the Hs68 fibroblast cell line.

The use of various bioassays has previously shown that exogenous IGFBPs, particularly IGFBP-3, can act to both inhibit and potentiate IGF-I activity, usually depending on the incubation protocol (summarised in the Introduction). By comparing the effect of  $\text{TNF}\alpha$ , which as we have shown inhibits IGFBP secretion, upon the mitogenic responses to either native IGF-I or to a synthetic IGF-I analogue with minimal affinity for the IGFBPs, we have now shown that *endogenous* IGFBPs appear to act in an inhibitory manner at the tissue level. Sensitivity of the cells to native IGF-I was increased approximately five-fold in the presence of  $\text{TNF}\alpha$ , an effect not observed with the IGF-I analogue, confirming it as one involving IGFBPs. Recent data from Cohen *et al* (1993) are in agreement with this conclusion, as they demonstrated similar inhibitory activity of an endogenous IGFBP to IGF-I mitogenesis in mouse Balb/c fibroblasts transfected with the human IGFBP-3 gene.

The sensitising effect of  $\text{TNF}\alpha$ , and potentially  $\text{IL-1}\beta$  also, may have implications in determining the metabolic disruption seen associated with these inflammatory cytokines in cachetic patients suffering from cancer or chronic infection. Both of these conditions produce well characterised changes in carbohydrate metabolism, typically including an enhanced rate of glucose utilisation by a number of tissues, that can be mimicked by intravenous administration of exogenous  $\text{TNF}\alpha$  (Lopez-Soriano *et al*, 1993). As it has also been demonstrated, experimentally at least, that  $\text{TNF}\alpha$  can lead to an impairment or resistance of insulin action on glucose disposal in peripheral tissues (Lang *et al*, 1992) it seems that another candidate for this effect other than insulin must exist. Although  $\text{TNF}\alpha$  itself may have some function in regulating glucose metabolism, perhaps its enhancement of IGF-I activity that we have observed in such isolated peripheral tissue (skin fibroblasts) via alterations in IGFBP secretion may contribute to this phenomenon.

Caution must be taken however in the interpretation of IGF bioactivity data recorded from simple *in vitro* bioassays such as that presented in this thesis, as due to their enclosed



undynamic nature they fail to take account of the aforementioned stability and trapping properties of the IGFBPs which may serve to *enhance* IGF action. Data gained from such assays may therefore be pertinent only to locally derived autocrine or paracrine IGFs, whose significance to whole body metabolism and growth is unclear. We must also consider the possible degree of cell-type specificity to the effect of TNF $\alpha$  and IL-1 $\beta$ , particularly as in a number of cell systems the IGFs seem to act in an opposing fashion to these cytokines. For example, a great deal of data now exists on the ability of TNF $\alpha$  and IL-1 to induce or aggravate the tissue destructive processes commonly associated with rheumatoid arthritis (Brennan *et al*, 1992). High concentrations of these inflammatory cytokines in the affected limb joints, resulting from infiltrating activated monocytes and macrophages, can lead to both local protease synthesis and the inhibition of essential connective matrix components, such as proteoglycans and hyaluronan, thus contributing greatly to the pathogenesis of the disease. The effect of IGF-I on these proteins is often quite the reverse, even antagonising some of the effects of the cytokines, and thus makes any extrapolation of our *in vitro* bioassay data to *in vivo* pathologies somewhat speculative. However, one could argue that if sensitisation to IGF-I by TNF $\alpha$  modulation of IGFBPs is highly restricted to fibroblasts then perhaps this may contribute to increased proliferation of this cell-type and the development of fibrosis often observed in the arthritic joint.

Although we cannot quantify the contribution that fibroblast-derived IGFBPs make to the circulating concentration of these proteins, the assumption that a cytokine-mediated fall in their secretion may lead to a parallel reduction in the serum component must bring with it a different interpretation of its effect upon IGF-I bioactivity than that given by our bioassay. As most (approximately 99%) of the IGFs in serum associate with IGFBPs, and most of these in the ternary IGFBP-3 complex, it may be particularly significant that the secretion of this IGFBP species is inhibited by TNF $\alpha$  and IL-1. The close correlation between IGFBP-3 and the IGFs may mean that the overall effect of these cytokines could be to lower the circulating IGF level, thus *reducing* its overall endocrine potential. Further studies are obviously required to investigate the effect of TNF $\alpha$  and IL-1 on hepatic IGFBP output, thought to be the primary source of circulating IGFBPs, although



it is tempting to hypothesize that a negative effect in serum may be at least partly responsible for some of the growth abnormalities and metabolic alterations often observed in a number of chronic inflammatory conditions. In juvenile chronic arthritis, for example, growth retardation is a common feature (Svantesson 1991). This is especially so in severe cases of the disease such as the systemic form with associated polyarthritis, where there is an acceleration of epiphysis formation in the active inflammatory joints infiltrated with cytokine-releasing macrophages and monocytes. Whilst there are a host of hormonal changes which may contribute towards the disruption of growth in these patients, such as elevated plasma levels of glucagon, epinephrine, glucocorticoids, adrenocorticotrophic hormone, and not least the use of corticosteroid therapy, recent evidence has reported that despite normal growth hormone secretory profiles the mean plasma IGF-I levels were abnormally low (Allen *et al*, 1991). This may be the result of course from altered hepatic gene expression of the growth factor, as has been observed in response to IL-1 $\beta$  in Leydig cell *in vitro* (Lin *et al*, 1992), but may also possibly be due to reduced IGFBP secretion. Further clinical studies will be required to establish the potential role that the inflammatory cytokines have in this effect.

Another clinical condition which has been associated with elevated cytokine production is in the development of graft-versus-host disease (GVHD) following bone marrow transplants in recipients with leukaemia (Holler *et al*, 1990). Excessive release of TNF $\alpha$  and IL-1 $\beta$  is thought to cause the induction of a shock-like state with consequent tissue injury, and an exaggerated and prolonged acute phase protein response (APPR) which involves redirecting body protein metabolism away from peripheral tissues towards the liver. Achieving positive nitrogen accretion in these patients, or indeed in others suffering critical surgical illness or cancer induced cachexia, is often not possible by the simple provision of calories and nitrogen. Recently, interest has arisen in the potential therapeutic use of IGFs in view of their well known ubiquitous protein sparing and anabolic actions and also from studies that have shown that IGF-I infusion into TNF $\alpha$  treated animals results in a marked reduction in protein loss (Douglas *et al*, 1991). High doses of insulin, perhaps operating through the type-I IGF receptors, have also been reported to reverse the toxic effects of this cytokine in a rat model (Fraker *et al*, 1989). Data showing



considerable reductions in serum IGFs and IGFBPs in human cachectic patients (Davies *et al*, 1991) may suggest that treatment in these animal models was successful because they simply replaced the 'lost' IGF fraction. Whether the low IGF concentration is due to cytokine mediated alterations in IGFBP abundance, or just follows the disruption of nutrient intake (known to inhibit IGF-I gene expression) remains to be established.

Whilst it is easier from a practical level to consider hormone, growth factor or cytokine activity on an isolated basis, it is almost certain that any pathological condition concerning TNF $\alpha$  and IL-1 involves a complex interaction between many of these components. For example, it is well recognised that signals leading to the release of TNF $\alpha$  and IL-1 may also stimulate the secretion of other cytokines, and even the IGFs themselves, from certain haematopoietic cells. One of these closely related peptides is interferon-gamma (IFN-g), which as we have shown in this thesis has the ability individually to inhibit IGFBP-3 secretion from our Hs68 fibroblasts. However, co-incubation of IFN-g with TNF $\alpha$  on these cells can result in the complete abolition of any IGFBP-3 response. Although this may add further confusion to the significance of the TNF $\alpha$  response, perhaps it may indicate that IFN-g acts as part of a homeostatic negative feedback mechanism, whereby IGFBP-3 secretion is brought back to normal levels. The fact that these particular cytokines appear to have developed such a counter-regulatory mechanism gives support to the hypothesis that their mediation of IGFBP-3 inhibition has some physiological and perhaps pathological significance.

### **9.3. The relationship of TGF $\beta$ to the IGFs**

The physiological role of TGF $\beta$  in regulating cell proliferation is generally held to be inhibitory, although it was first discovered as the factor produced by retrovirus-transformed fibroblasts which could support the anchorage-independent growth of normal cells. Since this initial discovery however, TGF $\beta$  has been shown to be a potent growth inhibitor of epithelial, endothelial, and lymphocyte cells, and may also antagonise platelet derived growth factor (PDGF) stimulation of fibroblasts. Following this logic, the stimulatory effect of TGF $\beta_1$  on Hs68 fibroblast IGFBP-3 secretion, via enhanced gene



expression, seems to fit with the view that this binding protein is inhibitory to IGF activity.

The involvement of TGF $\beta$  in modulating IGF-I mediated mitogenesis has been reported previously, with proliferation of T-47D human breast cancer cells being abolished by somewhat large doses ( $>10 \mu\text{g/l}$ ) of TGF $\beta_1$  and TGF $\beta_2$  (Zugmeier *et al*, 1989). Although IGFBP-3 was not measured in this study, such retardation of IGF activity could be consistent with an increase in IGFBPs. In our study, we have shown that TGF $\beta_1$  reduces the sensitivity of our normal human fibroblast cells to stimulation by IGF-I approximately ten-fold. No effect of TGF $\beta_1$  was seen on the activity of the LongR3-IGF-I analogue, which confirmed its inhibition of the native form as involving IGFBPs.

It was notable that only IGFBP-3 secretion was increased following TGF $\beta_1$  treatment, with similar reductions in IGFBP-4 and IGFBP-5 to that seen in response to TNF $\alpha$ . The opposing action of the two cytokines upon IGF-I activity in these cells suggests the importance of IGFBP-3 in these effects, although the TGF $\beta_1$ -induced reduction in the conditioned media levels of other IGFBPs may reflect their compartmentalisation into additional extracellular matrix (ECM) synthesised in response to TGF $\beta$ . This would provide additional inactive sites for localisation of IGF-I thus reducing its bioactivity further, although it should be noted that Jones *et al* (1993) failed to detect IGFBP-4 in foetal fibroblast ECM.

The opposing actions of TGF $\beta_1$  and TNF $\alpha$  (and IL-1) in regulating IGFBP-3 and consequently their modulating effects on IGF activity, were not wholly unexpected. Many actions of TGF $\beta$  appear to be diametrically opposed to those of TNF $\alpha$  and IL-1, and include positive effects upon bone formation, a general increase in the production and deposition of ECM components and the stimulation of connective tissue formation. Perhaps what is surprising in view of the negative effect of TGF $\beta_1$  on IGF-I bioactivity, is that these effects appear to parallel many of those shown by the IGFs themselves. TGF $\beta$  and IGF-I have been detected in significant quantities in wound sites and both have been shown to aid the healing process. Although one may suspect that locally increased IGFBP-3, in response to TGF $\beta$ , may be detrimental to IGF-I mediated wound healing there are reports that it may actually enhance the IGF effect. Recombinant IGFBP-3, for



example, has been shown to slightly increase wound healing when injected alone into the site of injury in rat tissue (Mueller *et al*, 1991), possibly by locally trapping IGFs. In addition, treatment with IGF-I together with equimolar amounts of IGFBP-3 was found to be twice as effective as the same dose of IGF-I alone in enhancing all aspects of wound healing. Although our bioactivity data seems to suggest that TGF $\beta_1$  can inhibit IGF-I activity via increased IGFBP-3 secretion, this may not necessarily be an accurate reflection of conditions *in vivo* therefore.

### SECTION C. Future Objectives

Perhaps the overriding message to be gained from the data presented in this thesis is the diversity and complexity of IGF / IGFBP physiology. Within a single cell type, in this instance human skin fibroblasts, at least three IGFBP species are produced and each may be stimulated or inhibited by a variety of factors such as the IGFs themselves and a number of cytokines. Regulation of IGFBPs may occur via one or more of a multitude of mechanisms, including alterations at the gene expression level or modulation of specific IGFBP protease activity. Additionally, the cytokine studies presented in this thesis do not take into account the complex interactions between these molecules themselves and their associated binding proteins, making the picture even more complex. Clearly then *in vitro* studies of this nature, although providing valuable information, are still very much in their infancy and a large number of questions remain as to their physiological implications.

Although in this thesis we have tried to address the question as to the effect of altering IGFBP secretion on subsequent IGF bioactivity, the use of a simple *in vitro* bioassay may be somewhat invalid. We do not know, for example, what is the relationship between circulating IGFBPs and those produced at the tissue level, although it is likely that hepatic IGF peptide, accounting for the vast majority in the vasculature, arrives at the cell already bound to IGFBPs. Obviously, our bioassay model cannot account for this. It may be that the action of autocrine / paracrine derived IGFs may be influenced more by variations in locally produced IGFBPs (such as that caused by cytokines for example), but we are unsure at present as to the overall importance of this source of IGF.

To answer some of these questions we now therefore plan to extend our studies to include some clinical models. In particular, we hope to establish the relationships between the cytokines and IGF / IGFBPs in pauciarticular Juvenile Chronic Arthritis patients. This model seems ideal to take our *in vitro* work further as it is characterised by the localisation of inflammatory cytokines to isolated joints, associated growth abnormalities, and compartmentalisation of the IGFs and IGFBPs between the circulation and synovial fluid. We eventually aim to investigate the regulation of local IGFBPs within the joints by *in situ* hybridisation and immunohistochemistry and to establish whether the cytokine effects described in this thesis occur *in vivo* and are pathologically implicated.



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